(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 October 2001 (25.10.2001)

PCT

(10) International Publication Number
WO 01/79499 A1

(51) International Patent Classification[7]: C12N 15/29, 15/53, 9/02, A01H 1/00, 5/00, 5/10

(21) International Application Number: PCT/AU01/00436

(22) International Filing Date: 18 April 2001 (18.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/198,124 18 April 2000 (18.04.2000) US

(71) Applicant *(for all designated States except US)*: COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2612 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GREEN, Allan [AU/AU]; 6/53 Elimatta Street, Braddon, ACT 2612 (AU). SINGH, Surinder [AU/AU]; 10 Lucas Place, Downer, ACT 2606 (AU). LIU, Qing [AU/AU]; 3/60 Dalley Crescent, Latham, ACT 2615 (AU).

(74) Agents: OLIVE, Mark, R. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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WO 01/79499 A1

(54) Title: METHOD OF MODIFYING THE CONTENT OF COTTONSEED OIL

(57) Abstract: The present invention provides novel gene constructs and methods for the production of transgenic cotton plants that produce oils having a range of desirable attributes, including improved oil quality, and modified fatty acid composition.

METHOD OF MODIFYING THE CONTENT OF COTTONSEED OIL

RELATED APPLICATION DATA

This application claims priority from USSN 60/198,124 filed on April 18, 2000.

FIELD OF THE INVENTION

The invention relates to a novel method of modifying the fatty acid composition of cottonseed oils to improve their nutritional and functional characteristics and to the plants, plant parts and metabolites produced therefrom.

BACKGROUND OF THE INVENTION

Cottonseed is a valuable product of cotton. Cottonseed contains approximately 25% of cottonseed oil, a well-established commodity vegetable oil because of its use as either a food ingredient or as a cooking oil for food preparation (Cherry, 1984). The world production of cottonseed oil in 1997/98 was around 4 million tonnes, making it sixth in importance behind the oils of soybean, oil-palm, rapeseed, sunflower and groundnut (Oil World Annual, 1998).

Globally, cotton crops consist of four domesticated *Gossypium spp.*, including the allotetraploid species *G. barbadense* L. and *G. hirsutum* L., and the diploid species *G. arboreum* L. and *G. herbaceum* L. Of these four species, *G. hirsutum* (upland cotton) is the predominant species, accounting for the overwhelming majority of cotton production worldwide. Currently, most cottonseed oil is derived from *G. hirsutum,* possibly as a consequence of the fact that this species is the major worldwide source of cotton fibre, and, cotton crops are primarily grown for their fibre.

The major components of cottonseed oil are the saturated fatty acids, palmitic acid (C16:0) and stearic acid (C18:0); the monounsaturated fatty acid, oleic acid (C18:1); and the polyunsaturated fatty acids, linoleic acid (C18:2) and α-linolenic acid (C18:3). A typical cottonseed fatty acid profile contains high levels of palmitic

acid (24%) and linoleic acid (54%), a moderate level of oleic acid (18%), and a very low level of stearic acid (3%) and α-linolenic acid (1%).

The number, position, and conformation of a double bond in each fatty acid present in the cottonseed influences the physical properties (such as melting temperature), chemical properties and nutritional value of the cottonseed oil, and the applications to which it may be put, particularly in the food industry.

For example, the presence of a carbon double bond in a monounsaturated fatty acid or polyunsaturated fatty acid lowers its melting temperature, compared to the melting temperature of a saturated fatty acid of the same carbon chain length, such that the C-18 unsaturated fatty acids, oleic acid, linoleic acid, and linolenic acid, are all liquid at ambient temperature.

Additionally, the susceptibility of a fatty acid to oxidation increases proportionately with the number of carbon double bonds present in the fatty acid molecule, dramatically reducing the suitability of oils containing polyunsaturated fatty acids to applications involving the use of prolonged heat in the presence of oxygen, such as cooking and other food service applications.

For applications that require solid fat components such as in solid cooking fats, shortenings, or margarines, it is necessary to have moderately high levels of saturated fatty acids, or the functionally equivalent *trans*- fatty acids. *Trans*-fatty acids have carbon double bonds in the *trans*-orientation rather than the naturally-occurring *cis*-orientation.

Currently, the unsaturated fatty acids are subjected to chemical hydrogenation, to improve their suitability in cooking and food service applications. Hydrogenated cottonseed oil is a valuable product, because cottonseed oil has a naturally-high level of palmitic acid, and desired melting properties can be readily achieved by the hydrogenation process. In this process, *trans*-fatty acids are produced as an artifact.

The nutritional quality of natural cottonseed oil, and hydrogenated cottonseed oil, has been questioned because of the reported adverse effects of both saturated fatty acids, and *trans* fatty acids (Wollett 1994). The contribution of high levels of some saturated fatty acids in the diet, particularly palmitic acid, to increased blood cholesterol, and more particularly to increased low density lipoprotein (LDL) , is well-established. Elevated LDL in the blood has been associated with an enhanced risk of cardiovascular disease in humans. Moreover, *trans*-fatty acids also elevate LDL cholesterol in a manner similar to palmitic acid. Because of the proven association with risk of cardiovascular disease, nutritionists and health authorities generally recommend limiting the dietary intake of palmitic acid, and *trans*-fatty acids, to at least below 30% of total fat intake. Natural oils high in palmitic acid, and hydrogenated oils high in *trans*-fatty acids, are expected to lose favour as a consequence of these recommendations.

However, not all saturated fatty acids are associated with elevated cholesterol. For example, stearic acid is reported to have neutral effects on blood cholesterol (Wollett 1994). in this respect, the high melting temperature of stearic acid (approximately 70°C) also makes it particularly suitable in solid fat applications. Accordingly, because of its neutral effects on blood cholesterol levels, a high stearic acid-containing oil is a desirable substitute for partially-hydrogenated plant oils currently used in margarine production. Because of its physicochemical properties, stearic acid is also suitable for use in the production of cosmetics, pharmaceuticals and candles (Topfer, 1995). Furthermore, novel cottonseed oil having approximately equal proportions of palmitic, stearic and oleic and therefore having considerable potential for use as a cocoa butter substitute.

Although polyunsaturated fatty acids are beneficial in terms of lipoprotein metabolism and cardiovascular health, they are highly susceptible to peroxidation.

In summary, because the major use of cotton seed oil is as a foodstuff, there is a need to develop improved oils that have enhanced human nutritional value, such

as, for example, oils that have reduced palmitic acid and/or are high in stearic acid. Furthermore, there is also a need to improve the potential applications of cottonseed oil in the food industry, without the adverse health risks associated with using oils rich in many saturated fatty acids, by providing oils having novel unsaturated fatty acid profiles. For example, oils having high oleic acid content and/or low linoleic acid content have the desired physicochemical properties of a cooking oil, and do not require hydrogenation (Kinney 1996).

Furthermore, triacylglycerol composed of approximately equal proportions of palmitic acid, oleic acid, and stearic acid (i.e. POS-type triacylglycerol) has a very sharp melting temperature at around body temperature, making it particularly suitable as a substitute for cocoa butter in the manufacture of chocolate and other confectionery. (Fincke, 1976; Gunstone *et al.*, 1986). Currently, the seeds of the cocoa tree, *Theobroma cacao* L., are the sole source of cocoa butter, and, as a consequence, cocoa butter is often in short supply and costly. The development of cocoa butter substitutes is of considerable economic importance.

In some cases, plant breeders have been able to modify the fatty acid content or composition of seed-derived oils, by inducing mutations in fatty acid biosynthesis genes. Exposure of plant material, generally seeds, to certain mutagenic agents, such as radiation or chemical mutagens, combined with traditional plant breeding approaches, has successfully produced a wide range of novel fatty acid profiles in many oilseed crops, including mutants of rapeseed (Auld *et al.*, 1992), sunflower (Soldatov, 1976) and soybean (Rahman *et al.*, 1994), having increased oleic acid; mutants of soybean (Erickson *et al.*, 1988), linseed (Rowland and Bhatty, 1990), and sunflower (Osorio *et al.*, 1995), having increased palmitic acid; mutants of soybean (Fehr *et al*, 1991) having lowered palmitic acid; mutants of soybean (Graef *et al.*, 1985; Rahman *et al.*, 1995) and sunflower (Osorio *et al.*, 1995)having increased stearic acid; and mutants of linseed (Green, 1986), soybean (Wilcox *et al.*, 1984), and rapeseed (Robbelen and Nitsch, 1975) having lowered linolenic acid. In several cases this has led to the commercial exploitation of these mutants, such as in the development of commercial varieties of high-oleate

sunflower (Miller *et al.*, 1987) and low-linolenic linseed (Green *et al.*, 1991) and rapeseed (Scarth *et al.*, 1988) oils. However, in spite of such notable successes in most other oilseed crops, there are no reports of substantial genetic modification of fatty acid composition in cottonseed oil using induced mutagenesis.

As a result of extensive basic biochemical research over a number of decades, the pathway for synthesis of the predominant fatty acids and their subsequent assembly into the seed storage triglycerides (oils) is now well understood for many plant species. Nearly all of the enzymes involved in fatty acid metabolism have been identified, the biosynthetic steps catalysed therefor characterised, and the genes encoding said enzymes cloned. In particular, the genes encoding stearoyl-ACP Δ9-desaturases, and oleoyl-ACP Δ12 desaturases have been cloned from several oilseed species, as follows.

The cDNAs encoding fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) enzymes from approximately 22 plant species, including castor bean (Shanklin and Somerville 1991) , safflower (Thompson *et al.*, 1991) , and cotton (Liu *et al.*, 1996) have been cloned, and the nucleotide sequences thereof made publicly available from the GenBank database. Antisense gene constructs comprising a nucleotide sequence complementary to the *Brassica rapa* stearoyl-ACP Δ9-desaturase cDNA have been used to decrease expression of the endogenous *B. napus* and *B. rapa* stearoyl-ACP Δ9-desaturase genes (Knutzon 1992), thereby increasing stearic acid at the expense of oleic acid in the seed oil. In this case, stearic acid was increased to 40% of total fatty acid in the seed.

With regard to fatty acid Δ12-desaturase genes, a cDNA containing the open reading frame of the *Arabidopsis thaliana FAD2* gene has been isolated, and shown to complement the *fad2* mutation of *A. thaliana*, which mutation produces a deficiency in the activity of the oleoyl-PC Δ12-desaturase enzyme (Miquel and Browse, 1992), indicating that the *FAD2* gene encodes an oleoyl-PC Δ12-desaturase (Okuley *et al.*, 1994). Kinney (1997) decreased expression of

endogenous rapeseed and soybean fatty acid Δ12-desaturase genes, by using sense-suppression (cosuppression) and antisense-suppression gene constructs, to produce high oleic acid-containing oils. In that work, Kinney (1997) also reported the decreased expression of fatty acid Δ15-desaturase genes, to produce low linolenic acid-containing oils in both rapeseed and soybean. Cosuppression to reduce expression of an endogenous fatty acid Δ12-desaturase gene has also been reported to produce high oleic acid oils in *Brassica napus* and *Brassica juncea* (Stoutjesdijk *et al.*,1999).

United States Patent No. 5, 850, 026 (Cargill, Inc.), dated 15 December, 1998, also reports the production of high oleic acid-containing oilseed in *Brassica sp.*, by using antisense or cosuppression gene constructs directed simultaneously against microsomal fatty acid Δ12-desaturase *and* microsomal fatty acid Δ-15 desaturase gene expression. The oilseed reported by these workers was also low in erucic acid and α-linolenic acid.

United States Patent No. 5, 981,781 (E.I. du Pont de Nemours and Company), dated 9 November, 1999, teaches the use of cosuppression, to reduce expression of the soybean *GmFAD2-1* gene, which encodes a fatty acid Δ12-desaturase (oleoyl-PC Δ12-desaturase) in that species. A high oleic acid-containing soybean oil, having high oxidative stability, was produced by this cosuppression.

More recently, Liu *et al.* (1999a, 1999b) have described a fatty acid Δ12-desaturase (oleoyl-PC Δ12-desaturase) gene from cotton.

Notwithstanding the considerable number of publicly-available plant fatty acid biosynthesis genes which have been cloned and characterised, and the reported modification of fatty acid levels in the oils of *Brassica spp.* and soybean using said genes, there is no reported modification of fatty acid metabolism in cotton, using

either traditional plant breeding, mutational breeding, or recombinant DNA approaches. The tetraploid nature of cotton, and the existence of large families of specific fatty acid biosynthesis genes makes it difficult to determine those genes which, by virtue of being expressed in a seed-specific manner are suitable targets for silencing with a view to modifying oil seed composition.

Additionally, gene silencing is not a straightforward procedure as applied to cotton. There are only a few reports in the literature of the transformation of cotton using gene silencing gene constructs, and these reports are restricted to the use of antisense technology. For example, antisense technology has been used to reduce the expression of genes involved in fibre synthesis, however in that report the transgenic plants did not exhibit a detectable phenotype notwithstanding a reduction in enzyme biosynthesis, suggesting that the silencing of genes in cotton is unpredictable

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of

exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Reference herein to prior art, including any one or more prior art documents, is not to be taken as an acknowledgment, or suggestion, that said prior art is common general knowledge in Australia or forms a part of the common general knowledge in Australia.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

This specification contains nucleotide sequence information prepared using the program PatentIn Version 3.0, presented herein after the claims. Each nucleotide sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier [e.g. <210>1, <210>2, etc]. The length, type of sequence [DNA, protein (PRT), etc] and source organism for each nucleotide sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide sequences referred to in the specification are defined by the term "SEQ ID NO:", followed by the sequence identifier [e.g. SEQ ID NO: 1 refers to the sequence in the sequence listing designated as <400>1].

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymidine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymidine, S represents Guanine or Cytosine, W represents Adenine or Thymidine, H represents a nucleotide other

than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymidine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

SUMMARY OF THE INVENTION

In work leading up to the present invention, the inventors sought to develop a modified cottonseed oil having improved nutritional and physicochemical characteristics, such as, for example, oils having improved oxidative stability and melting characteristics, without the need for hydrogenation, and oils having improved nutritional characteristics, by virtue of their modified fatty acid composition. More particularly, the inventors sought to develop specialty cottonseed oils which are low in palmitic acid and/or linoleic acid, and/or which are high in stearic acid and/or oleic acid, using a combination of recombinant DNA technology and classical plant breeding approaches. To achieve this objective, the inventors found it necessary to develop the means to reproducibly apply gene silencing approaches to cotton, and to identify specific fatty acid biosynthesis genes in cotton that are expressed in a seed-specific manner.

Accordingly, one aspect of the invention provides a method of modifying the endogenous oil of a cotton plant comprising producing a transgenic cotton plant having a gene construct which comprises a nucleotide sequence of a fatty acid biosynthesis gene or a gene fragment thereof, wherein said gene or gene fragment is placed operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant, and wherein said fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes, and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

In accordance with this aspect of the invention, the inventors have isolated nucleic acid molecules which comprise nucleotide sequences encoding these fatty acid

biosynthesis genes, such as, for example the nucleotide sequences of cotton exemplified herein as SEQ ID NOs: 1, 3, and 5. These nucleotide sequences have been used to develop novel gene constructs capable of producing over-expression, sense-suppression, cosuppression, antisense suppression, and post-transcriptional gene silencing (PTGS) of the various fatty acid genes of cotton.

The inventors have also isolated the genomic *ghFAD2-1* gene of cotton encoding the Δ12-desaturase (oleoyl-PC Δ12-desaturase) enzyme (Example 10). SEQ ID NO: 7 shows the nucleotide sequence of the *ghFAD2-1* promoter. In this respect, SEQ ID NO: 7 contains sufficient nucleotide sequence to confer expression on a structural gene to which it is operably connected in the cottonseed. In particular, the 5006 nucleotides of SEQ ID NO: 7 includes 3784 nucleotides upstream of the transcription start site of the *FAD2* gene (position 3785), the first intron of the gene (nucleotides 3889 to 4998) and the entire 5'-untranslated region (UTR) of the *ghFAD2-1* gene (nucleotides 3785 to 5006).

In particular, the inventors developed gene constructs to facilitate the silencing or reduced seed-specific expression of cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes. The gene constructs have been introduced into cotton explants to produce transformed cells that have subsequently been regenerated into whole plants. Surprisingly, the cottonseed oil produced by transgenic lines containing any one of the introduced gene constructs is low in palmitic acid compared to the oil of isogenic non-transformed lines. As exemplified herein, the level of palmitic acid in the seed oil of transformed cotton lines, is reduced to approximately 50% of the level of palmitic acid detected for non-transformed plants. Moreover, the transformed lines containing gene silencing constructs which are targeted against the cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes, and cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes, have high stearic acid, and high oleic acid, respectively, in the cottonseed oil, compared to isogenic non-transformed lines. As exemplified herein,

silencing of the cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene has produced cotton plants having as much as 15-fold the stearic acid content of non-transformed lines, with stearic acid accounting for up to approximately 40% of the total lipid of the oil. In elite lines having high oleic acid, this fatty acid accumulated at the expense of linoleic acid, with oleic acid accounting for as much as approximately 80% of total seed lipid.

Intermediate levels of these fatty acids may be obtained by modification of the strength of promoter used to regulate expression of the introduced fatty acid biosynthesis gene, and/or by the selection of specific transgenic lines having desired levels of a particular fatty acid in the oil, and/or by recombination of the phenotypes of particular plants such as by standard sexual hybridisation. Such procedures are well within the ken of a skilled person.

The inventors have further shown that it is possible to combine the high oleic acid and high stearic acid traits, and to obtain intermediate levels of these fatty acids, by conventional plant breeding of elite lines, preferably without compromise of the low palmitic acid characteristic.

Accordingly, a further aspect of the invention provides a cotton plant having increased oleic acid and stearic acid in the seed wherein said plant is produced by sexual hybridisation between a first cotton plant having increased oleic acid in the seed compared to an isogenic non-transformed cotton plant and a second cotton plant having increased stearic acid in the seed compared to an isogenic non-transformed cotton plant, and wherein said first cotton plant and/or said second cotton plant is/are produced in accordance with the inventive method of down-regulating expression of a gene selected from the group consisting of: cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes; and cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

In an alternative embodiment, this invention provides a cotton plant having decreased palmitic acid in the seed, wherein said plant is produced by sexual hybridisation between a first cotton plant and a second cotton plant, and wherein said first cotton plant and/or said second cotton plant is/are produced in accordance with the inventive method of down-regulating expression of a gene selected from the group consisting of: cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene and cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

A further aspect of the invention provides a transgenic cotton plant produced in accordance with the inventive method and having a fatty acid biosynthesis gene or a gene fragment thereof introduced into its genome, wherein said fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes. This aspect of the invention clearly extends to cotton plants consisting of the progeny of the primary transformant plants which comprise the introduced fatty acid biosynthesis gene or gene fragment. This aspect of the invention further extends to all plant parts, and, in particular, to seed derived from the primary transformed plant or its progeny. Preferably, the seed will comprise the introduced fatty acid biosynthesis gene or gene fragment and, more preferably, such seed will have an oil having a modified fatty acid composition in accordance with the invention (i.e. low palmitic acid and/or high oleic acid and/or high stearic acid and/or low linoleic acid).

This invention clearly extends to any and all of the gene constructs used in the performance of the inventive method as described herein. In particular, this aspect of the invention provides a gene construct comprising the nucleotide sequence of a fatty acid biosynthesis gene, or a gene fragment thereof, placed operably in connection with a promoter sequence that is operable in cotton seed, wherein said gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9

stearoyl-ACP desaturase) genes, and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

Data provided herein indicate that gene silencing constructs comprising inverted repeats of both coding and non-coding regions of fatty acid desaturase genes can be used to effectively modulate cottonseed oil composition. Additionally, the gene construct may comprise an inverted repeat sequence that is disrupted, such as, for example, by the inclusion of an intron sequence between the inverted repeats, to effectively modulate cottonseed oil composition. Moreover, gene constructs comprising inverted repeats of non-coding sequences, particularly 5'-non-coding sequences, disrupted (i.e. interrupted) by an intervening sequence are particularly useful because they produce an enhanced number of primary transformants having modified fatty acid composition relative to untransformed controls.

Data provided herein show further that the invention can be performed using diverse promoter sequences to regulate expression of the gene silencing construct in cottonseed. The *ghFAD2-1* gene promoter and the soybean lectin gene promoter are particularly preferred.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation showing cotton seed oil content, and fatty acid composition, in developing cotton seed embryos. Samples of cottonseed embryos at 15 to 65 DAA were collected and analysed.

Figure 2 is a schematic representation showing the nucleotide and deduced amino acid sequences of the ghSAD-1 cDNA clone encoding the cotton stearoyl-ACP Δ9-desaturase polypeptide (SEQ ID NO: 1). The translation start site ATG is underlined. An asterisk indicates the stop codon. Two iron-binding motifs,

separated by about 100 amino acids, are indicated in bold type. The arrow indicates a putative transit peptide cleavage site. The fatty acid desaturase family II signature motif is also underlined.

Figure 3 is a copy of a photographic representation of a Northern blot hybridisation of developing cotton seed embryos, and cotton leaf tissue. Panel (A) shows an ethidium bromide-stained RNA gel. Panel (B) shows the hybridisation signal obtained using a [α-^{32}P]dCTP-labelled ghSAD-1-specific probe. Lanes 1-4 represent RNA samples isolated from embryos at 25, 30, 36, and 45 DAA, respectively. Lane 5 represents RNA isolated from young leaves.

Figure 4 is a copy of a photographic representation of a Southern blot hybridisation of cotton genomic DNA, probed with nucleotide sequences derived from the Δ9 stearoyl-ACP desaturase cDNA clone ghSAD-1. Panel (A) shows the genomic DNA fragments hybridising to the 3'-UTR of ghSAD-1. Panel (B) shows the genomic DNA fragments hybridising to the coding region of ghSAD-1. Lane 1, *G. barbadense* DNA; Lane 2, *G. hirsutum* cv Deltapine-16 DNA; Lane 3, *G. hirsutum* cv Siokra DNA; Lane 4, *G. herbaceum* DNA; and Lane 5, *G. raimondii* DNA.

Figure 5 is a schematic representation showing the nucleotide and deduced amino acid sequences of the ghFAD2-1 cDNA clone encoding the cotton oleoyl-PC Δ12-desaturase polypeptide (SEQ ID NO: 3). The translation start site (ATG) is in bold type. The priming site for the oligonucleotide *Δ12*A4, which was used as a PCR primer to amplify the 5' end of the gene, is underlined, and the arrow indicates the 5' to 3' direction of the primer. The asterisk indicates the stop codon.

Figure 6 is a schematic representation showing the nucleotide and deduced amino acid sequences of the 1422 bp-long ghFAD2-2 cDNA clone encoding the cotton

oleoyl-PC Δ12-desaturase polypeptide. The translation start site (ATG) is in bold type. The asterisk indicates a stop codon.

Figure 7 is a schematic representation of an amino acid sequence alignment between ghFAD2-1 (SEQ ID NO: 4), *Glycine max* microsomal ω-6 desaturase (gmFAD2-2; SEQ ID NO: 31), A. *thaliana* microsomal ω-6 desaturase (atFAD2; SEQ ID NO: 32), *G.max* microsomal ω-6 desaturase (gmFAD2-1; SEQ ID NO: 33), *B. napus* microsomal ω-3 desaturase (bnFAD3; SEQ ID NO: 34), and *G. max* plastid ω-6 desaturase (gmFAD6; SEQ ID NO: 35). Residues indicated in bold type are amino acids that are conserved among all sequences. Shaded areas indicate homologous residues in one or more of the different desaturase sequences. Dots indicate spaces introduced to maximise alignments. Three histidine boxes proposed to be important in iron binding are underlined. Six glycine repeats in relative C-terminus of the ghFAD2-1 polypeptide are also underlined.

Figure 8 is a schematic representation of a Northern blot hybridisation of developing cotton seed embryos, and cotton leaf tissue. Panel (A) shows an ethidium bromide-stained RNA gel. Panel (B) shows the hybridisation signal obtained using a [α-^{32}P]dCTP-labelled ghFAD2-1-specific probe. Panel (C) shows the hybridisation signal obtained using a [α-^{32}P]dCTP-labelled ghFAD2-2-specific probe. Lanes 1-4 represent RNA samples isolated from embryos at 25, 30, 36, and 45 DAA, respectively. Lane 5 represents RNA isolated from young leaves.

Figure 9 is a copy of a photographic representation of a Southern blot hybridisation of cotton genomic DNA, probed with nucleotide sequences derived from the oleoyl-PC Δ12 desaturase cDNA clone ghFAD2-1. Panel (A) shows the genomic DNA fragments hybridising to the 3'-UTR of ghFAD2-1. Panel (B) shows the genomic DNA fragments hybridising to the 3'-UTR of ghFAD2-2. Panel (C) shows the genomic DNA fragments hybridising to the coding region of ghFAD2-1. Lane 1, *G. barbadense* DNA; Lane 2, *G. hirsutum* cv Deltapine-16 DNA; Lane 3,

G. hirsutum cv Siokra DNA; Lane 4, *G. herbaceum* DNA; Lane 5, *G. raimondii* DNA; and Lane 6, G. *robinsonii.*

Figure 10 is a schematic representation of the binary vector pBI121, and the soybean lectin gene promoter and terminator of pGLe-10 which were used to produce the pBI-Lectin binary vector.

Figure 11 is a graphical representation showing the frequency distribution of stearic acid in 15 individual seeds of Coker cotton (upper panel) and 60 individual T2 seeds derived from 4 T1 transgenic cotton plants comprising an inverted repeat of the 5′-end of the ghSAD-1 cDNA clone (lower panel).

Figure 12 is a graphical representation showing the frequency distribution of oleic acid in 15 individual seeds of Coker cotton (upper panel) and 75 individual T2 seeds derived from 5 T1 transgenic cotton plants comprising an inverted repeat of the 5′-end of the ghFAD2-1 cDNA clone (lower panel).

Figure 13 is a graphical representation showing the relationship between stearic acid (x-axis) and oleic acid (abscissa) content, as a percentage of total fatty acids, in 86 F2 seeds derived from the 125-23 x 95-150 cross that are classified as high oleic acid seed (○). high stearic acid seed (▲), high stearic acid and high oleic acid seed (♦), or normal Coker 315-type seed (◇). Data show that the levels of both of these fatty acids can be modulated to produce combinations of high stearic and high oleic acid that are not found in either transformed parental line or in the untransformed Coker 315 parent.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the invention provides a method of modifying the endogenous oil of a cotton plant comprising producing a transgenic cotton plant having a gene construct which comprises a nucleotide sequence of a fatty acid biosynthesis gene or a gene fragment thereof, wherein said gene or gene fragment is placed operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant, and wherein said fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes, and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

For the present purpose, "cotton" is to taken as referring to any plant belonging to the genus *Gossypium* and to any wild relatives, progenitor species, germplasm, cultivar or variety thereof, and to any derivative germplasm, cultivar or variety therefrom.

Without limiting the scope of the present invention, the cotton species to which the inventive method is applicable can be any species selected from the group consisting of: *G. anapoides, G. anomalum, G. arboreum, G. areysianum, G. aridum, G. armourianum, G. australe, G. barbadense, G. barbosanum, G. benadirense, G. bickii, G. briccetti, G. capitis-viridis, G. costulatum, G. cunninghamii, G. darwinii, G. davidsonii, G. enthyle, G. exiguum, G. gossypioides, G. harknessii, G. herbaceum, G. hirsutum, G. incanum, G. klotzschianum, G. laxum, G. lobatum, G. londonderriense, G. longicalyx, G. marchantii, G. mustelinum, G. nandewarense, G. nelsonii, G. nobile, G. pilosum, G. populifolium, G. pulchellum, G. raimondii, G. robinsonii, G. rotundifolium, G. schwendimantii, G. somalense, G. soudanense, G. stocksii, G. sturtianum, G. thurberi, G. timorense, G. tomentosum, G. trilobum, G. triphyllum,* and *G. viridis.*

Those skilled in the art will be aware that a cotton "variety" is a cotton plant that is contained within a single botanical taxon of the lowest known rank, such as, for example, a plant belonging to a particular species, or a hybrid of two species, wherein said plant expresses one or more stable characteristics that distinguish it from any other group or grouping. By "cultivar" is meant a cultivated variety. In the present context, "germplasm" shall be taken to mean one or more phenotypic characteristics, or one or more genes encoding said one or more phenotypic characteristics, capable of being transmitted between generations.

As the inventive method relates to the modification of cotton oils, it is not necessary for the cotton to be a variety which is commercially successful or of economic utility by virtue of its oil content and/or composition. Conveniently, the cotton upon which the inventive method is applied is a cultivated variety or cultivar or germplasm belonging to a *Gossypium sp.* selected from the group consisting of *G. barbadense, G. hirsutum, G. tomentosum, G. arboreum* and *G. herbaceum,* or a derivative species, germplasm or variety therefrom.

As used herein, the term "progenitor" shall be taken to refer to any of the species, varieties, cultivars, or germplasm, from which a plant is derived. As will be known to those skilled in the art, most commercially useful cotton is a naturally-occurring allotetraploid derived by sexual hybridisation between ancient diploid progenitor parents. Based upon the teaching provided herein, those skilled in the art can readily perform the inventive method on one or more diploid or allotetraploid cottons and produce sexual hybrids between the transgenic plants produced therefrom. The present invention clearly encompasses such alternatives.

As used herein, the term "derivative species, germplasm or variety" shall be taken to mean any plant species, germplasm or variety that is produced using a stated cotton species, variety, cultivar, or germplasm, using standard procedures of sexual hybridisation, recombinant DNA technology, tissue culture, mutagenesis, or

a combination of any one or more said procedures. For example, those skilled in the art are aware that there is compatibility between varieties or cultivars within the species *G. barbadense, G. hirsutum,* and *G. tomentosum,* of which *G. barbadense,* and *G. hirsutum* are of particular economic significance. In particular, interspecific hybrids have been produced between *G. barbadense,* and *G. hirsutum*, and between certain diploid species, and the production of such interspecific hybrids is routine to those skilled in the art.

Preferred elite cultivars of cotton include any of the major Australian and United States cultivars, such as, for example, Siokra L22, Siokra L23, Siokra L23i, Siokra V15i, Sicala V2, Sicala V2i, Sicala 33, Sicala 34, Sicot 189i, CS50, Acala R, Suregrow125, Suregrow501, Suregrow404, Deltapine50, Deltapine51, DES56, DES119, DES24, Delcott277, Auburn 257-202, Stoneville 603, Stoneville 213, PD2164, Frego25, and Arkot 8110, amongst others, or a derivative thereof. Of these cultivars, at least Siokra L22, Siokra L23, Sicala 33, and Sicala 34 normally contain relatively high levels of palmitic acid and linoleic acid, and moderate amounts of mono-unsaturated fatty acids. Accordingly, it is a particularly preferred object of the present invention to reduce the level of palmitic acid, and/or to increase the level of stearic acid and/or oleic acid, in these cultivars.

It is a routine matter for those skilled in the art to produce sexual hybrids between two or more of cotton species, varieties, cultivars, or germplasms, and to subsequently modify the oil composition of said sexual hybrids based upon the teaching provided herein. Accordingly, the inventive method clearly includes the further first step of producing such sexual hybrids.

In accordance with the inventive concept, the transgenic cotton plant has a modified endogenous oil. This means that the total endogenous oil content of the cotton plant, in particular the oil content of the seed, is increased or decreased relative to an isogenic non-transformed plant, or that the composition of said oil is

modified relative to an isogenic non-transformed plant, such as, for example, by increasing or decreasing the content of any particular fatty acid in the oil.

Preferably, the relative content of one or more fatty acids in cotton seed oil is increased or decreased, and, more preferably, the content of one or more C16:0 and/or C18:0 fatty acids is modified.

Even more preferably, the inventive method provides the means for modifying (i.e. increasing or decreasing) the content of a fatty acid selected from the group consisting of palmitic acid, oleic acid, linoleic acid, and stearic acid.

In a particularly preferred embodiment of the invention, the inventive method provides the means for modifying the composition of cotton seed oil wherein the modification consists of a modified fatty acid composition selected from the group consisting of:

(i) decreased palmitic acid content of the oil relative to the oil of a non-transformed isogenic cotton plant;

(i) increased stearic acid content of the oil relative to the oil of a non-transformed isogenic cotton plant;

(i) increased oleic acid content of the oil relative to the oil of a non-transformed isogenic cotton plant; and

(i) decreased linoleic acid content of the oil relative to the oil of a non-transformed isogenic cotton plant.

The present invention clearly extends to any and all effects on oil content and/or composition which are derived from producing a transgenic cotton plant having one or more fatty acid biosynthesis genes or gene fragments introduced into its genome, and, in particular, to those effects which include a reduction in the level of palmitic acid in the cotton seed oil. As exemplified herein, transgenic cotton having

a cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene or gene fragment introduced into its genome produces an oil having decreased palmitic acid and increased stearic acid relative to the oil of a non-transformed isogenic cotton plant, whilst transgenic cotton having a single cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene or gene fragment introduced into its genome produces an oil having decreased palmitic acid, decreased linoleic acid, and increased oleic acid relative to the oil of a non-transformed isogenic cotton plant. Accordingly, the present invention clearly extends to any and all combinations of the modified fatty acid compositions listed hereinabove.

As used herein, the term "relative to a non-transformed isogenic cotton plant", or similar term, shall be taken to mean that a stated integer has a value attributed to it by virtue of a comparison with the value of that integer obtained under similar or identical conditions from a non-transformed cotton plant of the same species as that from which the transgenic cotton plant was derived. Preferably, such a comparison is made by reference to starting plant material from which the transgenic cotton plant was derived or plant material that has the same oil content and composition as said starting plant material.

The oil content of cotton seed, or the content of any fatty acid in the oil of a cotton seed, can be conveniently determined as a proportion of the total lipid of the seed. Procedures for determining the lipid content of cotton seed, and for determining the content and/or composition of fatty acids in cotton seed oil, are well-known to those skilled in the art. For example, the total lipid content of cottonseed or seed oil may be determined using the procedure described by Folsch *et al.* (1957) or the modification of that procedure described herein. The fatty acid content and/or composition of cotton seed oil may be conveniently determined using gas liquid chromatography against known standard fatty acids, by comparing the fatty acid methyl ester peaks and retention times of the standards with the sample being tested, and by standard integration of the peaks obtained. However, the present invention is not to be limited by the method of determining the content and/or

composition of cotton seed oil, in particular the means for determining fatty acid or other lipid components.

In accordance with the inventive concept, a cotton plant as described hereinabove, including any progenitor or derivative species, is modified by the introduction of a fatty acid biosynthesis gene or a fragment thereof, to produce a transgenic plant. It will be apparent from the preceding discussion that, for the purposes of the modification of cotton seed oil in accordance with the inventive concept, the fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

For the purposes of nomenclature, the terms "fatty acid Δ9-desaturase" and "Δ9 stearoyl-ACP desaturase" shall be taken to refer to refer to a peptide, polypeptide, protein or enzyme which is capable of producing a carbon double bond at the C-9 position of a saturated C18:0 fatty acid, to form a C18:1-ACP (i.e. oleoyl-ACP) fatty acid.

The terms "fatty acid Δ12-desaturase", and "oleoyl-PC Δ12-desaturase" shall be taken to refer to refer to a peptide, polypeptide, protein or enzyme which is capable of producing a carbon double bond at the C-12 position of a mono-unsaturated C18:1 fatty acid (i.e. oleoyl-PC), to form a C18:2-PC (i.e. linoleoyl-PC) fatty acid.

Reference herein to a "gene" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5′- and 3′- untranslated sequences);or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene; or

(iii) amplified single-stranded or double-stranded DNA which is derived from sub-paragraph (i) or sub-paragraph (ii).

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product, which may be derived from a naturally-occurring gene, mRNA, or cDNA, by standard recombinant techniques, including any additional nucleotide sequences derived from a homologous or heterologous source which may added to (i) or (ii) or (iii) or said synthetic or fusion molecules.

In the present context, the term "fatty acid biosynthesis gene" or similar term refers to any gene which, in its native context at least, comprises a nucleotide sequence which is capable of encoding a fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase), or fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) as hereinbefore defined.

In the present context, the term "gene fragment" shall be taken to mean a part of a full-length fatty acid biosynthesis gene as hereinbefore defined, wherein said part is of a suitable length, orientation, and conformation, to be capable of modulating the expression of an endogenous cotton fatty acid biosynthesis gene when introduced into, and preferably expressed in, a cotton oil-producing organ, such as, for example, the seed, including any direct or inverted repeat sequences derived from said gene. Accordingly, the term "part" clearly includes any single fragment of the full-length gene in the sense or antisense orientation, and any inverted repeat sequences having partial or complete self-complementarity, the only requirement being that such parts are capable of modulating the expression of an endogenous fatty acid biosynthesis gene when introduced to cotton. With particular regard to inverted repeat sequences, such sequences will further qualify as parts of the full-length gene whether or not the repeated sequences are

contiguous, or alternatively, whether or not the repeated sequences are interrupted by one or more intervening nucleotide sequences, such as, for example, one or more intron sequences. Those skilled in the art will readily be capable of determining whether or not a partial gene or gene fragment is of a suitable length, orientation, and conformation for the purposes of modifying cotton seed oil, without undue experimentation, by empirical means following the teaching provided herein.

For the purposes of further defining the fatty acid biosynthesis gene and gene fragments thereof which are used in performing the inventive method, there are provided herein nucleotide sequences which encode, or are complementary to nucleotide sequences which encode fatty acid biosynthesis enzymes, and gene fragments of said nucleotide sequences and complementary nucleotide sequences, wherein said fatty acid biosynthesis enzymes are selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) enzymes as hereinbefore defined.

Wherein it is desired to increase expression of an endogenous cotton fatty acid biosynthesis gene in order to modify oil composition of the seed, such as, for example, by ectopically-expressing a heterologous or foreign cotton fatty acid biosynthesis gene in cotton seed, the introduced nucleotide sequence is preferably capable of being expressed in cotton at the protein level. Accordingly, in such an embodiment of the invention, it is particularly preferred that the introduced nucleotide sequence will have a codon usage in any protein-encoding part thereof which is suitable for translation in a cotton plant. In those embodiments of the invention wherein it is desired to decrease expression of an endogenous cotton fatty acid biosynthesis gene in order to modify oil composition of the seed, it is preferred that there is sufficient complementarity between the mRNAs encoded by the introduced gene and the endogenous gene so as to hinder, prevent, or reduce the expression of the endogenous gene in the cotton seed, such as, for example,

by hybridisation between said mRNAs in the nucleus and/or cytoplasm. As will be apparent to those skilled in the art, any improvements which flow from using introduced fatty acid biosynthesis genes having the codon usage preferences of cotton, and/or high nucleotide sequence identity with an endogenous cotton gene, can be conveniently provided by using nucleotide sequences derived from cotton. However, the present invention is not to be limited by the use of cotton fatty acid biosynthesis genes and gene fragments thereof.

For the purposes of nomenclature, the nucleotide sequence set forth in SEQ ID NO: 1 relates to a cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene, designated ghSAD-1, comprising a 1553 bp cDNA clone obtained by the present inventors, and encoding the fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) polypeptide set forth herein as SEQ ID NO: 2.

The nucleotide sequence set forth in SEQ ID NO: 3 relates to a first cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene, designated ghFAD2-1, comprising a 1411 bp cDNA clone obtained by the present inventors, and encoding the fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) polypeptide set forth herein as SEQ ID NO: 4.

The nucleotide sequence set forth in SEQ ID NO: 5 relates to a second cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene, designated ghFAD2-2, comprising a 1422 bp cDNA clone obtained by the present inventors, and encoding the fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) polypeptide set forth herein as SEQ ID NO: 6.

The nucleotide sequence set forth in SEQ ID NO: 7 relates to the nucleotide sequence of the genomic *ghFAD2-1* gene that extends upstream 5006 bp from the translation start site of the gene, but not including said translation start site.

Accordingly, in a particularly preferred embodiment, the present invention encompasses the use of a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 7, or any homologous nucleotide sequences, or degenerate nucleotide sequence thereto, or a complementary nucleotide sequence thereto or a gene fragment thereof, to modify oil in cotton.

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which encodes a polypeptide having the same enzyme activity as that encoded by a stated nucleotide sequence, notwithstanding the occurrence within said homologous sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements relative to the stated nucleotide sequence. Accordingly, the present invention clearly extends to the use of homologues of the nucleotide sequences set forth herein, including any homologues of complementary nucleotide sequences and homologues of any degenerate nucleotide sequences thereto. Particularly preferred homologues are those fatty acid biosynthesis genes and gene fragments derived from species other than cotton.

For example, there are numerous examples of nucleotide and amino acid sequences for cloned plant Δ9-desaturase genes lodged in the GenBank database (Table 1), and the sequence similarity between these genes (Shanklin, 1998; Mekhedov et al., 2000) is sufficient to facilitate the isolation of the equivalent homologous genes from other plant species by heterologous probing and/or amplification. To determine putative homologues of cotton Δ9-desaturase genes, a multiple alignment is performed between the derived amino acid sequences encoded by cloned plant Δ9-desaturase genes, such as, for example, the genes listed in Table 1, to identify regions of maximum conservation. Degenerate oligonucleotides are then reverse-engineered based upon the consensus amino

acid sequence of one or more of such highly-conserved amino acid sequences. In one approach to isolate cotton homologues, cotton gene libraries (eg seed-specific cDNA or genomic DNA libraries) are probed directly using the oligonucleotides under low stringency hybridisation conditions. Alternatively, the oligonucleotides are used as PCR primers to amplify putative Δ9-desaturase fragments from seed mRNA, or from an equivalent seed-specific cDNA library of cotton. Partial Δ9-desaturase gene fragments obtained in this way are then used as probes to isolate full-length gene sequences from the cDNA library. Alternatively, or in addition, a partial or full-length nucleotide sequence obtained from an already-cloned plant Δ9-desaturase gene can be used to directly probe a cotton gene library. In the case of a multigene family, such as the cotton Δ9-desaturase gene family, the major seed-expressed gene(s) is then determined by analysing the expression pattern of the gene in a range of tissues, including the seed, using RT-PCR, northern hybridisation analysis, nuclear run-on, or nuclear run-off methods, amongst others.

Similar approaches are used to isolate seed-expressed homologues of known cloned plant Δ12-desaturase genes lodged in the GenBank database (Table 2).

By "degenerate nucleotide sequence" is meant a nucleotide sequence having the same protein-encoding capacity as a nucleotide sequence set forth in SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 5.

Preferred gene fragments suitable for use in the performance of the inventive method comprise at least about 50 nucleotides derived from the full-length gene sequence, more preferably at least about 100 nucleotides in length, and even more preferably at least about 350 nucleotides in length. In a particularly preferred embodiment, the fatty acid biosynthesis gene used in performing the inventive method comprises the protein-encoding nucleotide sequence of a naturally-occurring fatty acid biosynthesis gene, or a complementary nucleotide sequence

thereto. Such gene fragments may comprise about 500 to about 850 nucleotides in length, for example, derived from the protein-encoding region of a fatty acid biosynthesis gene.

Additional gene fragments are not excluded, the only requirement being that such gene fragments are capable of being used to successfully modulate the expression of an endogenous cotton fatty acid biosynthesis gene.

TABLE 1

List of microsomal Δ9-desaturase genes and related sequences

Plant Species	Accession No.	Reference
Arabidopsis thaliana	X93461	GenBank X93461
Arachis hypogaea	AF172728	Tate *et al.*, 1999
Brassica napus	X63364	Slocombe *et al.*, 1992
Brassica juncea	AF153420	Vageeshbau *et al.*, 1999
Brassica rapa	X60978	Knutzon *et al.*, 1992
Elaeis guineensis	U68756	Shah and Rashid, 1996
Sesamum indicum	D42086	Yukawa *et al.*, 1994
Carthamus tinctorius	M61109	Thompson *et al.*, 1991
Coriandrum sativum	M93115	Cahoon *et al.*, 1992
Cucumis sativa	M59858	Shanklin *et al.*, 1991
G. hirsutum	AJ132636	Liu *et al.* 2000
G. hirsutum	AI730379	Blewitt *et al.*, 1999
Glycine max	L34346	Chen and Moon, 1995
Helianthus annuus	U70374	GenBank U70374
Linum usitatissimum	X90762	Singh *et al.*, 1994
Linum usitatissimum	AJ006957	Jain, 1998
Linum usitatissimum	AJ0069578	Jain, 1998
Asclepias syriaca	U60277	Cahoon *et al.*, 1997
Olea europaea	U58141	Baldoni *et al.*, 1996
Pelargonium xhortorum	U40344	Schultz *et al.*, 1996
Persea americana	AF116861	Madi and Pruskey, 1999
Oryza sativa	D38953	Akagi *et al.*, 1995
Ricinus communis	M59857	Shanklin *et al.*, 1991
Sesamum indicum	D42086	Yukawa et al., 2000
Solanum commersonii	X78935	GenBank X78935
Simmondsia chinensis	M83199	Sato *et al.*, 1992
Spinacia oleracea	X62898	Nishida *et al.*, 1992

TABLE 1 cont...

Solanum tuberosum	M91238	Taylor *et al.*, 1992
T. alata pTAD2	U07597	Cahoon *et al.*, 1994
T. alata pTAD3	U07605	Cahoon *et al.*, 1994
T. alata Δ6-desaturase	U09269	Cahoon *et al.*, 1994

TABLE 2

List of microsomal Δ12-desaturase genes and related sequences

Plant species	cDNA or gene	Accession Number	Reference
Arabidopsis thaliana	cDNA	L26296	Okuley et al., 1994
Arachis hypogaea	cDNA	AF030319	Jeong et al., 1997
Arachis hypogea	cDNA	AF030319	Jeong et al., 1994
Borago officinalis	cDNA	AF074324	Sayanova et al 1998
Brassica carinata	cDNA	AF124360	Marillia *et al.*, 1999
Brassica juncea	cDNA	X91139	Singh et al., 1995
Brassica oleracea	cDNA	AF181726	Fourmann et al 1999
Brassica rapa	cDNA	AF042841	Tanhuanpaa 1999
Crepis alpina	cDNA	Y16285	Lee et al., 1998
Crepis palaestina	cDNA	CPY16284	Lee et al., 1998
Glycine max	cDNA	L43920	Heppard et al., 1996
Glycine max	cDNA	L43921	Heppard et al., 1996
G. hirsutum	cDNA	X97016	Liu et al., 1999
G. hirsutum	cDNA	Y10112	Liu et al., 1999
Helianthus annuus	cDNA	U91341	Hongtrakul et al 1997
Impatiens balsamina	cDNA	AF182520	Cahoon et al., 1999
Lactuca sativa	cDNA	AF162199	Lee et al., 1999

TABLE 2 cont...

Lesquerella fendleri	cDNA	AF016104	Broun et al., 1998
Momordica charantia	cDNA	AF182521	Cahoon et al., 1999
Mucor rouxii	cDNA	AF161219	Passorn et al., 1999
Petroselinum crispum	cDNA	U86072	Somssich et al., 1997
Prunus armeniaca	cDNA	AF071892	Mbeguie-A-Mbeguie
Richnus communis	cDNA	U22378	Van der Loo, 1995
Solanum commersonii	cDNA	X92847	Grillo, 1995
Vernonia galamensis	cDNA	AF188263	Hage et al., 1999
Vernonia galamensis	cDNA	AF188264	Hage et al., 1999
G. arboreum	gene	AJ244914	Liu *et al.*, 2000
G australe	gene	AJ244901	Liu *et al.*, 2000
G. australe	gene	AJ244902	Liu *et al.*, 2000
G. australe	gene	AJ244903	Liu *et al.*, 2000
G. barbadense	gene	AJ244918	Liu *et al.*, 2000
G. barbadense	gene	AJ244919	Liu *et al.*, 2000
G. bickii	gene	AJ244904	Liu *et al.*, 2000
G. bickii	gene	AJ244905	Liu *et al.*, 2000
G. bickii	gene	AJ244906	Liu *et al.*, 2000
G. costulatum	gene	AJ244889	Liu *et al.*, 2000
G. cunninghamii	gene	AJ244890	Liu *et al.*, 2000
G. darwinii (A)	gene	AJ244920	Liu *et al.*, 2000
G. darwinii (D)	gene	AJ244921	Liu *et al.*, 2000
G. enthyle	gene	AJ244891	Liu *et al.*, 2000
G. exiguum	gene	AJ244892	Liu *et al.*, 2000
G. gossypioides	gene	AJ244912	Liu *et al.*, 2000
G. herbaceum	gene	AJ244915	Liu *et al.*, 2000
G. hirsutum (A)	gene	AJ244922	Liu *et al.*, 2000
G. hirsutum (D)	gene	AJ244923	Liu *et al.*, 2000
G. klotzschianum	gene	AJ244910	Liu *et al.*, 2000
G. londonderriense	gene	AJ244893	Liu *et al.*, 2000

TABLE 2 cont...

G. marchantii	gene	AJ244894	Liu *et al.*, 2000
G. mustelinum (A)	gene	AJ244924	Liu *et al.*, 2000
G. mustelinum(D)	gene	AJ244925	Liu *et al.*, 2000
G. nelsonii	gene	AJ244907	Liu *et al.*, 2000
G. nelsonii	gene	AJ244908	Liu *et al.*, 2000
G. nobile	gene	AJ244895	Liu *et al.*, 2000
G. pilosum	gene	AJ244896	Liu *et al.*, 2000
G. populifolium	gene	AJ244897	Liu *et al.*, 2000
G. pulchellum	gene	AJ244898	Liu *et al.*, 2000
G. raimondii	gene	AJ244913	Liu *et al.*, 2000
G. robinsonii	gene	AJ244884	Liu *et al.*, 2000
G. robinsonii	gene	AJ244885	Liu *et al.*, 2000
G. rotundifolium	gene	AJ244899	Liu *et al.*, 2000
G. somalense	gene	AJ244916	Liu *et al.*, 2000
G. species novum	gene	AJ244900	Liu *et al.*, 2000
G. stocksii	gene	AJ244917	Liu *et al.*, 2000
G. sturtianum	gene	AJ244886	Liu *et al.*, 2000
G. sturtianum	gene	AJ244887	Liu *et al.*, 2000
G. sturtianum	gene	AJ244888	Liu *et al.*, 2000
G. tomentosum (A)	gene	AJ244926	Liu *et al.*, 2000
G. tomentosum (D)	gene	AJ244927	Liu *et al.*, 2000
G. trilobum	gene	AJ244909	Liu *et al.*, 2000
G. turneri	gene	AJ244911	Liu *et al.*, 2000

By "expression" is meant transcription and/or translation, with or without subsequent post-translational events which modify the biological activity, cellular or sub-cellular localization, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

As used herein, the term "modulate the expression", or similar term, shall be taken to mean that the expression of a stated integer is enhanced, increased or decreased, or that the expression of a stated integer is delayed, inhibited or activated. Such a modulation of expression may be evidenced by direct assay of the integer, such as, for example, by comparison of signals obtained in a northern hybridisation, RT-PCR, or other means to measure steady state levels of mRNA, or alternatively, by comparing protein levels in the cell using ELISA or other immunoassay, SDS/PAGE, or enzyme assay. Alternatively, the modulation of expression may be evidenced by a modificaiton in the phenotype associated with the stated integer, such as, for example, by determining the fatty acid composition of the oil.

In the present context, the term "modulate the expression of an endogenous cotton fatty acid biosynthesis gene" shall be taken to mean that the expression of a fatty acid biosynthesis gene present in a cotton plant is modified, and/or that the enzyme activity of a fatty acid biosynthesis enzyme in cotton is modified, notwithstanding that such an effect may be produced by the ectopic expression of a heterologous (i.e. foreign) or introduced nucleotide sequence in cotton, or by increasing the copy number of cotton fatty acid biosynthesis genes, the only requirement being that such effects are directly or indirectly attributable to the presence of an introduced fatty acid biosynthesis gene or gene fragment thereof.

Those skilled in the art will be aware of whether gene expression has been modified by performance of the invention in cotton, without undue experimentation. For example, the level of expression of a particular gene may be determined by polymerase chain reaction (PCR) following reverse transcription of an mRNA template molecule, essentially as described by McPherson *et al.* (1991).

Alternatively, the expression level of a genetic sequence may be determined by northern hybridisation analysis or dot-blot hybridisation analysis or *in situ* hybridisation analysis or similar technique, wherein mRNA is transferred to a membrane support and hybridised to a probe" molecule which comprises a nucleotide sequence complementary to the nucleotide sequence of the mRNA transcript encoded by the gene-of-interest, labelled with a suitable reporter molecule such as a radioactively-labelled dNTP (eg [α-^{32}P]dCTP or [α-^{35}S]dCTP) or biotinylated dNTP, amongst others. Expression may then be determined by detecting the appearance of a signal produced by the reporter molecule bound to the hybridised probe molecule. Alternatively, the rate of transcription of a particular gene may be determined by nuclear run-on and/or nuclear run-off experiments, wherein nuclei are isolated from a particular cell or tissue and the rate of incorporation of rNTPs into specific mRNA molecules is determined. Alternatively, expression of a particular gene may be determined by RNase protection assay, wherein a labelled RNA probe or riboprobe" which is complementary to the nucleotide sequence of mRNA encoded by said gene is annealed to said mRNA for a time and under conditions sufficient for a double-stranded mRNA molecule to form, after which time the sample is subjected to digestion by RNase to remove single-stranded RNA molecules and in particular, to remove excess unhybridised riboprobe. Such approaches are described in detail by Sambrook *et al.* (1989) and Ausubel (1987). Those skilled in the art will also be aware of various immunological and enzymatic methods for detecting the level of expression of a particular gene at the protein level, for example using rocket immunoelectrophoresis, ELISA, radioimmunoassay and western blot immunoelectrophoresis techniques, amongst others.

Preferably, the fatty acid biosynthesis gene or gene fragment is in a format suitable for the ectopic expression of a fatty acid biosynthesis polypeptide in cotton, or alternatively, for the silencing or down-regulation of expression of an endogenous fatty acid biosynthesis gene in cotton.

As will be known to those skilled in the art, to ectopically express a polypeptide, the structural gene region which encodes said polypeptide is placed in the sense orientation in operable connection with a suitable promoter sequence so as to provide for transcription and translation in the cell. In the present context, as such ectopic expression is intended to lead to an increase in the activity of a fatty acid biosynthesis polypeptide, it is essential for the structural gene region to encode a polypeptide having enzymatic activity in cotton seed. Accordingly, the present invention encompasses the use of the nucleotide sequences set forth herein or any fragments thereof which encode functional enzymes, and any degenerate nucleotide sequences thereto, or homologous gene sequences derived from other species.

Whilst not being bound by any theory or mode of action, means for silencing or otherwise reducing the expression of an endogenous fatty acid biosynthesis gene in cotton include means which target transcription and/or mRNA stability and/or mRNA turnover and/or accessibility of mRNA to ribosomes or polysomes. Such means include sense suppression, cosuppression, antisense suppression, ribozyme-mediated gene silencing, and post-transcriptional gene silencing (PTGS). As with the ectopic expression of genes, it is preferred to introduce the nucleic acid in operable connection with a promoter sequence for the purposes of silencing or otherwise reducing the expression of an endogenous fatty acid biosynthesis gene in cotton.

Sense suppression and cosuppression of gene expression utilise nucleotide sequences which are positioned in the sense orientation in an expressible format (i.e. "sense molecules") As used herein, the term "sense molecule" shall be taken to refer to an isolated nucleic acid molecule which encodes or is complementary to an isolated nucleic acid molecule which is capable of encoding a fatty acid biosynthesis polypeptide, including full-length polypeptides having enzyme activity and fragments thereof lacking enzyme activity, wherein said nucleic acid molecule is provided in a format suitable for expression when introduced into a cotton plant by transfection or transformation.

As used herein, the term "co-suppression" shall be taken to mean a reduction in expression of an endogenous cotton fatty acid biosynthesis gene that occurs when one or more copies of said gene, or one or more copies of a substantially similar gene are introduced into the cell. The present invention clearly extends to the use of co-suppression to inhibit the expression of an endogenous fatty acid biosynthesis gene in cotton.

In "sense suppression", expression of the endogenous fatty acid biosynthesis gene is decreased by virtue of expressing in the cell a dominant-negative sense molecule. For example, a sense molecule, which encodes an enzymatically-inactive fatty acid biosynthesis polypeptide, may be expressed in the seed. Alternatively, a sense molecule which encodes a partial or full-length mRNA species may be introduced to the cotton genome such that transgene insertion events lead to a reduction in expression of both the endogenous gene and the transgene. Preferred dominant-negative sense molecules will comprise at least one or more functional protein domains of the wild-type protein, such as, for example, a domain which is involved in dimerisation with other polypeptide subunits of a functional holoenzyme.

In the context of the present invention, the term "antisense suppression" shall be taken to mean a suppression of gene expression which is mediated by expressing an antisense molecule in the cotton plant. An "antisense molecule" is an RNA molecule which is produced by transcription of the DNA strand which is complementary to that which is normally transcribed to produce a "sense" mRNA molecule capable of being translated into a fatty acid biosynthesis polypeptide. The antisense molecule is therefore complementary to the sense mRNA, or a part thereof. Although not limiting the mode of action of the antisense molecules of the present invention to any specific mechanism, the antisense RNA molecule possesses the capacity to form a double-stranded mRNA by base pairing with the sense mRNA, which may prevent translation of the sense mRNA and subsequent synthesis of a functional fatty acid biosynthesis polypeptide.

Ribozymes are synthetic RNA molecules which comprise a hybridising region complementary to two regions, each of at least 5 contiguous nucleotide bases in the target sense mRNA. In addition, ribozymes possess highly specific endoribonuclease activity, which autocatalytically cleaves the target sense mRNA. A complete description of the function of ribozymes is presented by Haseloff and Gerlach (1988) and contained in International Patent Application No. WO89/05852. The present invention extends to ribozymes which target a sense mRNA encoding an endogenous cotton fatty acid biosynthesis polypeptide, such that it is no longer capable of being translated to synthesise a functional polypeptide product.

In the context of the present invention, post-transcriptional gene silencing (PTGS) is a reduction in gene expression which is produced by introducing nucleic acid to the cell which comprises a first nucleotide sequence capable of being transcribed into sense mRNA, linked in head-to-head or tail-to-tail configuration, with or without any intervening nucleotide sequences, to a second nucleotide sequence capable of being transcribed into antisense mRNA such that the complete mRNA molecule which is transcribed from said nucleic acid comprises an inverted repeat sequence having self-complementarity. Whilst not being bound by any theory or mode of action, the transcript has the potential to form a secondary structure, such as, for example, a hairpin loop, in the nucleus and/or cytosol of a cell, and to sequester sense mRNA which is transcribed therein, such that single-stranded regions of the sequestered mRNA are rapidly degraded and/or a translationally-inactive complex is formed. The nucleotide sequences comprising the inverted repeat may include the full-length sequence of the target gene, or a gene fragment thereof, including both coding and/or untranslated nucleotide sequences.

As exemplified herein, highly-efficient PTGS may be performed using gene fragments of about 90 nucleotides in length, or longer, derived from the 5'-coding or non-coding end of the target gene, linked in head-to-head or tail-to-tail configuration. However, the present invention clearly encompasses the use of

inverted repeat sequences derived from fragments of less than 90 nucleotides in length, and derived from regions other than the 5'-end of the target gene. In particular, the present invention contemplates the use of gene fragments of only about 25 nucleotides in length or longer, derived from the 5'-end or 3'-end of the target nucleotide sequence, such as, for example, 25 bp derived from the 5'-UTR or 3'-UTR of the ghSAD-1 or ghFAD2-1 cDNA. As will be apparent to the skilled person, sequences longer than 90 nucleotides in length are also useful in performing the invention.

Moreover, the present invention clearly extends to the use of inverted repeats which are either contiguous inverted repeats of gene fragments, or alternatively, wherein each gene fragment comprising the inverted repeat is separated, or interrupted, by the insertion of one or more intervening nucleotide sequences. The use of intron sequences to space or interrupt each of the gene fragments comprising the inverted repeat is particularly encompassed by the invention. The intron sequence which is used in this embodiment may be any intron sequence, and preferably, albeit not necessarily, a plant intron sequence. As exemplified herein, the inventors have utilized an inverted repeat of about 90 nucleotides from the 5'-untranslated region of the endogenous *ghFAD2-1* gene wherein the repeat is disrupted by the complete intron-1 sequence of the gene.

The present invention clearly extends to the use of other gene fragments in performing the inventive method than those specifically exemplified herein. Preferred gene fragments for use in antisense and/or PTGS approaches will comprise a nucleotide sequence consisting of at least about 10 to 20 nucleotides of the target fatty acid biosynthesis gene, more preferably at least about 50-100 nucleotides, or a full-length or substantially full-length mRNA transcript encoded by said target gene.

It is understood in the art that certain modifications, including nucleotide substitutions, may be made to the genes and gene fragments used in performing the inventive method, without destroying the efficacy of said molecules in

modulating the expression of an endogenous fatty acid biosynthesis gene. It is therefore within the scope of the present invention to include any nucleotide sequence variants, homologues, or analogues of the said genes and gene fragments. The genes and gene fragments having utility in the inventive method will preferably comprise a nucleotide sequence having at least about 60-70% identity, more preferably at least about 70-80% identity, still more preferably at least about 80-90% identity or at least about 95-99% identity to the nucleotide sequence of a fatty acid biosynthesis gene which is set forth in any one or more of SEQ ID NOs: 1, 3, or 5 or 7, or a gene fragment thereof, or a complementary nucleotide sequence thereto.

Reference herein to a percentage identity or percentage similarity between two or more nucleotide or amino acid sequences shall be taken to refer to the number of identical or similar residues in a nucleotide or amino acid sequence alignment, as determined using any standard algorithm known by those skilled in the art. In particular, nucleotide and/or amino acid sequence identities and similarities may be calculated using the GAP program, which utilises the algorithm of Needleman and Wunsch (1970) to maximise the number of residue matches and minimise the number of sequence gaps. The GAP program is part of the Sequence and Analysis Software Package of the Computer Genetics Group Inc., University Research Park, Madison, Wisconsin, United States of America (Devereux *et al.*, 1984). In nucleotide and amino acid sequence comparisons which contain no gaps, the percentage identity may be calculated from a direct comparison of the number of identical nucleotides or amino acids there between, as the case may be, expressed as a percentage of the total number of nucleotides or amino acids in the sequences.

In an alternative embodiment, the genes and gene fragments used in performing the inventive method will preferably comprise a nucleotide sequence which is capable of hybridizing under at least low stringency conditions, more preferably under at least moderate stringency conditions and even more preferably under at

least high stringency conditions, to any one or more of SEQ ID NOs: 1, 3, 5 or 7, or a gene fragment thereof, or a complementary nucleotide sequence thereto.

For the purposes of defining the level of stringency, those skilled in the art will be aware that several different hybridisation conditions may be employed. For example, a low stringency may comprise a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C or room temperature. A moderate stringency may comprise a hybridisation and/or wash carried out in 2xSSC buffer, 0.1% (w/v) SDS at a temperature in the range 45°C to 65°C. A high stringency may comprise a hybridisation and/or wash carried out in 0.1xSSC buffer, 0.1% (w/v) SDS or Church Buffer at a temperature of at least 65°C. Variations of these conditions will be known to those skilled in the art.

Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS in the hybridisation buffer or wash buffer and/or increasing the temperature at which the hybridisation and/or wash are performed. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification of parameters affecting hybridisation between nucleic acid molecules, reference can conveniently be made to pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

The introduction of a fatty acid biosynthesis gene, or a gene fragment thereof, into cotton and expression may be facilitated by providing said gene or gene fragment in operable connection with a suitable promoter sequence, in the form of a gene construct or vector molecule. Accordingly, the present invention clearly extends to the use of gene constructs and vectors designed to facilitate the introduction and/or expression of the introduced genes and gene fragments in cotton, and particularly, in cotton seed.

In the present context, the term "gene construct" refers to any nucleic acid molecule that comprises one or more foreign nucleic acid molecules comprising

the nucleotide sequence of the fatty acid biosynthesis gene and/or a fragment thereof, in a form suitable for introducing into a plant cell, tissue, organ, or plant part, including a plantlet, and preferably which is capable of being integrated into the genome of a plant.

As used herein, the word "vector" shall be taken to refer to a linear or circular DNA sequence which includes a gene construct as hereinbefore defined, and which includes any additional nucleotide sequences to facilitate replication in a host cell and/or integration and/or maintenance of said gene construct or a part thereof in the host cell genome.

Preferred vectors include plasmids, cosmids, plant viral vectors, and the like, such as, for example, a plasmid or cosmid containing T-DNA to facilitate the integration of the foreign nucleic acid into the plant genome, such as, for example, binary transformation vectors, super-binary transformation vectors, co-integrate transformation vectors, Ri-derived transformation vectors, suitable for use in any known method of transforming cotton.

The term "vector" shall also be taken to include any recombinant virus particle or cell, in particular a bacterial cell or plant cell, which comprises the gene construct of the invention. For example, a plant virus, such as a gemini virus, amongst others, may be engineered to contain the fatty acid biosynthesis gene or gene fragment thereof, or alternatively, a gene construct containing the fatty acid biosynthesis gene or gene fragment may be introduced into *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, for subsequent transfer to cotton as described hereinabove.

In a particularly preferred embodiment, the gene construct contains the fatty acid biosynthesis gene or gene fragment thereof cloned within a binary transformation vector, such as, for example, the binary transformation vector pBI121 which is well-known to those skilled in the art to be suitable for *Agrobacterium*-mediated transformation by virtue of the presence of the T-DNA left and right border

sequences, and the *NPTII* structural gene placed operably in connection with the nopaline synthase promoter sequence which confers resistance on plant cells carrying the plasmid to the antibiotic kanamycin.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner, the only requirement being that said promoter sequence is capable of conferring expression on a fatty acid biosynthesis gene or gene fragment as described herein, in a cotton plant, and more particularly, in cotton seed.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of said sense molecule in cotton. Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. A promoter is usually, but not necessarily, positioned upstream or 5′ of a nucleic acid molecule which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene or gene fragment the expression of which it regulates. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene

from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Promoters suitable for use in genetic constructs of the present invention include promoters derived from the genes of viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants which are capable of functioning in cotton. The promoter may confer expression constitutively throughout the plant, or differentially with respect to the cotton seed, or differentially with respect to the developmental stage of the seed at which expression occurs, or in response to external stimuli such as physiological stresses, pathogens, or metal ions, amongst others.

Exemplary promoters suitable for use in the inventive method are listed in Table 3. The promoters listed in Table 3 are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention, and, in particular, the promoter of any fatty acid biosynthesis gene of a plant, and preferably a dicotyledonous plant. The present invention particularly contemplates the use of promoters derived from genes encoding fatty acid desaturases; fatty acid hydroxylases; fatty acid epoxygenases; fatty acid acetylenases; fatty acid conjugases; acyl carrier protein (ACP); acyl-ACP thioesterases; acyl transferases, acyl elongases; and fatty acid keto-acyl synthases. In a particularly preferred embodiment, the promoter sequence is the promoter region of the soybean lectin gene (Vodkin *et al.*, 1983; Cho *et al.*, 1995; GenBank Accession No. K00821) or the promoter of the cotton Δ12 desaturase gene (i.e. *ghFAD2-1* gene promoter) set forth in SEQ ID NO: 7, or a fragment of said promoter that is operable in cottonseed.

In addition to the fatty acid biosynthesis gene or gene fragment, and the promoter sequence, the gene constructs used in performing the inventive method will generally comprise a terminator sequence. The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the gene constructs of the present invention include the nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens,* the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the soybean lectin gene terminator, the Rubisco small subunit (SSU) gene terminator sequences and subclover stunt virus (SCSV) gene sequence terminators, amongst others. In a particularly preferred embodiment of the invention, the fatty acid biosynthesis gene or gene fragment thereof is placed upstream of the soybean lectin gene terminator. Those skilled in the art will be aware of additional terminator sequences which may readily be used without any undue experimentation.

TABLE 3

EXEMPLARY PROMOTERS FOR USE IN THE PERFORMANCE OF THE PRESENT INVENTION

I: CELL-SPECIFIC, TISSUE-SPECIFIC, AND ORGAN-SPECIFIC PROMOTERS		
GENE SOURCE	**EXPRESSION PATTERN**	**REFERENCE**
seed-specific genes	seed	Simon et al., 1985; Scofield et al., 1987; Baszczynski et al., 1990.
Brazil Nut albumin	seed	Pearson et al., 1992.
legumin	seed	Ellis et al., 1988.
glutelin (rice)	seed	Takaiwa et al., 1986; Takaiwa et al., 1987.
zein	seed	Matzke et al, 1990
napA	seed	Stalberg et al, 1996.
wheat LMW and HMW glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2, 1989
wheat SPA	seed	Albani et al,1997
wheat α, β, γ-gliadins	endosperm	EMBO 3:1409-15, 1984
barley ltr1 promoter	endosperm	
barley B1, C, D, hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750-60, 1996
barley DOF	endosperm	Mena et al, 1998

blz2	endosperm	EP99106056.7
synthetic promoter	endosperm	Vicente-Carbajosa et al., 1998.
rice prolamin NRP33	endosperm	Wu et al, 1998
rice α-globulin Glb-1	endosperm	Wu et al, 1998
rice OSH1	embryo	Sato et al,1996
rice α-globulin REB/OHP-1	endosperm	Nakase et al.,1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorghum γ-kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	embryo	Postma-Haarsma et al, 1999
rice oleosin	embryo and aleurone	Wu et at,1998
sunflower oleosin	seed (embryo and dry seed)	Cummins et al., 1992
soybean lectin	seed	Vodkin *et al.*, 1983; Cho *et al.*, 1995; GenBank Accession No. K00821
oleate Δ12-hydroxylase:desaturase	seed	Broun *et al.*, 1998
linseed promoters	seed	Jain *et al.*, 1999
palmitoyl-ACP thioesterase	cotton seed	Yoder *et al.*, 1999

II: EXEMPLARY CONSTITUTIVE PROMOTERS		
GENE SOURCE	**EXPRESSION PATTERN**	**REFERENCE**
Actin	constitutive	McElroy et al, 1990
CaMV 35S	constitutive	Odell et al, 1985
CaMV 19S	constitutive	Nilsson et al., 1997
GOS2	constitutive	de Pater et al,1992
ubiquitin	constitutive	Christensen et al, 1992
rice cyclophilin	constitutive	Buchholz et al, 1994
maize H3 histone	constitutive	Lepetit et al, 1992
actin 2	constitutive	An et al, 1996

The gene constructs of the invention may further include an origin of replication sequence required for replication in a specific cell type, for example a bacterial cell, when said gene construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell.

Preferred origins of replication include, but are not limited to, the *f1*-ori and *col*E1 origins of replication.

The gene construct may further comprise a selectable marker gene or genes that are functional in a cell into which said gene construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene and luciferase gene, amongst others.

To facilitate the *Agrobacterium*-mediated introduction of the fatty acid biosynthesis gene or gene fragment into cotton, it is particularly preferred for the introduced gene sequences including any promoter and terminator sequences, and with or without any selectable marker gene sequences or prokaryotic origins of replication, to be flanked by one or more T-DNA sequences. Preferred T-DNA sequences include one or more left and/or right border sequences derived from the *Agrobacterium tumefaciens* Ti plasmid.

The gene constructs are introduced into a cotton cell using standard procedures, and the transfected or transformed cell is subsequently regenerated to produce a transgenic cotton plant. By "transgenic plant" is meant a plant having foreign DNA introduced thereto by means of transfection or transformation. In the present context, the term "transgenic plant" shall be taken to include a cell, tissue or organ which is derived from a transgenic plant, and/or a cell, tissue or organ which capable of clonal propagation to produce a transgenic plant.

By "transfection" is meant that the process of introducing a gene construct or vector or an active fragment thereof which comprises foreign nucleic acid into a cell, tissue or organ derived from a plant, without integration into the cell's genome.

By "transformation" is meant the process of introducing a gene construct or vector or an active fragment thereof which comprises foreign nucleic acid into a cell, tissue or organ derived from a plant, wherein said foreign nucleic acid is stably integrated into the genome.

In the present context, "foreign nucleic acid " means any nucleic acid that is not present in the genome of the cell, tissue, or organ into which the gene construct or vector is introduced, and, in particular nucleic acid comprising the nucleotide sequence of a fatty acid biosynthesis gene or a fragment thereof in a format suitable for modifying the oil of cotton in accordance with the inventive method.

Means for introducing recombinant DNA into cotton tissue or cells will be known to those skilled in the art, and include, but are not limited to, direct DNA uptake into protoplasts (Krens *et al.*, 1982; Paszkowski *et al.*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al.* 1990), microparticle bombardment, electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.* 1986), microparticle

bombardment of tissue explants or cells (Christou *et al.*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, T-DNA-mediated transfer of *Agrobacterium* to the plant tissue as described essentially by An *et. al.* (1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985), or by Cousins *et al.* (1991).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed plant cell, tissue or organ. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.*, (US Patent No. 5,122,466) and Sanford and Wolf (US Patent No. 4,945,050). When using ballistic transformation procedures, the gene construct may incorporate a plasmid capable of replicating in the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole cotton plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (eg., apical meristem, axillary buds, and root meristems), and induced meristem tissue (eg., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

A particularly preferred method of producing a transgenic cotton plant is by *Agrobacterium*-mediated transformation of cotyledons, followed by the induction of callus formation, and the subsequent induction of embryogenic callus, and regeneration into plants, essentially as exemplified herein.

The regenerated transformed cotton plants described herein may take a variety of forms, such as, for example, chimeras of transformed cells and non-transformed cells; or clonal transformants (eg., all cells transformed to contain the expression cassette). They may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

Preferably, the introduced fatty acid biosynthesis gene or gene fragment is capable of being expressed in the cotton seed so as to modify (i.e. increase or decrease) the level of expression of an endogenous cotton fatty acid biosynthesis gene to a level that is sufficient to modify the content and/or composition of the oil produced in said seed.

The activity of a fatty acid biosynthesis enzyme in cotton seed oil may be determined using procedures known to those skilled in the art. For example, the enzyme activity may be calculated by direct enzyme assay, or alternatively, by indirect means involving the determination of substrate and product levels, such as, for example, by an appropriate algorithm which calculates the proportion of the fatty acid product of the enzyme as a proportion of the combined content of all such fatty acid products and remaining fatty acid substrate.

In a preferred embodiment, the activity of the fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) enzyme in cotton seed oil is determined using the Oleic Desaturation Proportion (ODP) algorithm, which determines the proportion of unsaturated polyunsaturated C18 fatty acids relative to the total unsaturated C18 fatty acids in cotton seed oil, as follows:

ODP= (C18:2 + C18:3) / (C18:1 + C18:2 + C18:3).

The ODP measures the proportion of total unsaturated C18 fatty acid that is desaturated by the fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) enzyme.

Alternatively or in addition, the activity of the fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) enzyme in cotton seed oil is determined using the Stearic Desaturation Proportion (SDP) algorithm, which determines the proportion of unsaturated C18 fatty acids relative to the total C18 fatty acids in cotton seed oil, as follows:

SDP= (C18:1 + C18:2 + C18:3) / (C18:0 + C18:1 + C18:2 + C18:3).

The SDP measures the proportion of total C18 fatty acid that is desaturated by the fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) enzyme.

It will be apparent from the preceding discussion that there are several steps involved in producing a transgenic plant, including production of gene constructs, transformation of a cotton cell with the gene constructs, the production of embryogenic callus from the transformed cotton cells, selection of transformed cells, regeneration into whole plants, and propagation of the transgenic plants by asexual or sexual means. The present invention clearly encompasses any and all of these steps collectively or individually, by the term "producing a transgenic cotton plant".

The inventive method described herein may further include the sexual hybridisation of the transgenic plant produced as described with any other transgenic or non-transgenic cotton species, variety, cultivar or germplasm, such as, for example, for the purpose of combining the desirable characteristics of both parents in the progeny of that sexual hybridisation. The present invention encompasses the use of the primary transgenic cotton plant, or the progeny thereof produced by self-fertilisation or out-crossing, as both a pollen parent or a seed parent.

Accordingly, the present invention clearly extends to a method of modifying the endogenous oil of a cotton plant comprising:

(i) producing a transgenic cotton plant having a gene construct which comprises a nucleotide sequence of a fatty acid biosynthesis gene or a gene fragment thereof, wherein said gene or gene fragment is placed operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant, and wherein said fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes; and

(ii) sexually hybridising said transgenic cotton plant with a second cotton plant to produce a progeny plant.

In accordance with this embodiment of the invention, the second cotton plant may be a transgenic cotton plant which produces a high level of stearic acid in the seed, by virtue of the presence and/or expression of one or more introduced fatty acid biosynthesis genes or gene fragments, such as, for example, an introduced fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene or gene fragment in the antisense orientation, or alternatively, an inverted repeat of a fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene or gene fragment which is capable of self-complementarity.

In an alternative embodiment, the second cotton plant is a transgenic cotton plant which produces a high level of oleic acid in the seed, by virtue of the presence and/or expression of one or more introduced fatty acid biosynthesis genes or gene fragments, such as, for example, an introduced fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene or gene fragment in the antisense orientation, or alternatively, an inverted repeat of a fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene or gene fragment which is capable of self-complementarity.

In a still further embodiment, the second cotton plant is a transgenic cotton plant which produces a low level of palmitic acid in the seed, by virtue of the presence and/or expression of one or more introduced fatty acid biosynthesis genes or gene fragments thereof, such as, for example:

(i) an introduced fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene or gene fragment in the antisense orientation; or alternatively,

(ii) an introduced fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene or gene fragment in the antisense orientation; or alternatively,

(iii) an inverted repeat of a fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene or gene fragment which is capable of self-complementarity; or alternatively,

(iv) an inverted repeat of a fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene or gene fragment which is capable of self-complementarity.

In a particularly preferred embodiment of the invention, sexual hybridisation is performed between a first cotton plant having increased oleic acid in the seed compared to an isogenic non-transformed cotton plant and a second cotton plant having increased stearic acid in the seed compared to an isogenic non-transformed cotton plant. Preferably, the progeny of such a crossing produces a seed oil which is high in oleic acid and stearic acid compared to an isogenic non-transformed cotton plant.

In an alternative embodiment, sexual hybridisation is performed between a first cotton plant having increased stearic acid in the seed compared to an isogenic non-transformed cotton plant and a second cotton plant having decreased palmitic acid in the seed compared to an isogenic non-transformed cotton plant. Preferably, the progeny of such a crossing produces a seed oil which is high in stearic acid and low in palmitic acid compared to an isogenic non-transformed cotton plant.

In a further alternative embodiment, sexual hybridisation is performed between a first cotton plant having increased oleic acid in the seed compared to an isogenic non-transformed cotton plant and a second cotton plant having decreased palmitic acid in the seed compared to an isogenic non-transformed cotton plant. Preferably, the progeny of such a crossing produces a seed oil which is high in oleic acid and low in palmitic acid compared to an isogenic non-transformed cotton plant.

In yet a further alternative embodiment, sexual hybridisation is performed between a first cotton plant having increased oleic acid and low palmitic acid in the seed compared to an isogenic non-transformed cotton plant and a second cotton plant having increased oleic acid and decreased palmitic acid in the seed compared to an isogenic non-transformed cotton plant. Preferably, the progeny of such a crossing produces a seed oil which is high in oleic acid and stearic acid, and low in palmitic acid, compared to an isogenic non-transformed cotton plant.

In yet a further alternative embodiment, sexual hybridisation is performed between a first cotton plant having decreased linoleic acid in the seed compared to an isogenic non-transformed cotton plant, and a second cotton plant having increased stearic acid in the seed compared to an isogenic non-transformed cotton plant. Preferably, the progeny of such a crossing produces a seed oil which is high in stearic acid and low in linoleic acid, compared to an isogenic non-transformed

cotton plant. More preferably, the progeny is also low in palmitic acid and/or high in oleic acid, compared to an isogenic non-transformed cotton plant.

The present invention clearly extends to all such alternatives. Other sexual hybridisations are not excluded.

The present invention clearly contemplates a sexual hybridisation between the transgenic cotton plant produced in accordance with the inventive method, and a naturally occurring cotton species, variety, cultivar, or germplasm which exhibits desirable oil-producing attributes, the only requirement being that said naturally occurring plant is compatible with the transgenic plant. For example, *G. arboreum* contains high levels of oleic acid in the seed, and reduced linoleic acid, of which both characteristics are desirable attributes.

The inventive method described herein further contemplates the genetic modification of a primary transgenic cotton plant or the progeny thereof to further modify the cotton seed oil. As with the use of sexual hybridisation, additional genetic modification may be used to introduce additional oil-modifying characteristics to the cotton plant, to further enhance its oil quality compared to the primary transgenic plant. In a particularly preferred embodiment, the present invention contemplates a method of modifying the endogenous oil of a cotton plant comprising:

(i) producing a first transgenic cotton plant having a gene construct which comprises a nucleotide sequence of a first fatty acid biosynthesis gene or a gene fragment thereof, wherein said gene or gene fragment is placed operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant, and wherein said first fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes;

(ii) sexually hybridising said first transgenic cotton plant with a cotton plant to produce a progeny plant; and

(iii) producing a second transgenic cotton plant having a gene construct which comprises a nucleotide sequence of a second fatty acid biosynthesis gene or a gene fragment thereof, wherein said gene or gene fragment is placed operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant, and wherein said second fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

Preferably, the second fatty acid biosynthesis gene or gene fragment is different to the first fatty acid biosynthesis gene or gene fragment.

Those skilled in the art will be aware that the same end-product may be obtained by changing the series in which this procedure is conducted, or by using the second transgenic plant, rather than the first transgenic plant as a seed or pollen parent. Accordingly, the present invention clearly encompasses such alternatives.

A further aspect of the present invention provides a transgenic cotton plant having modified oil composition, and more particularly, modified content and/or composition of one or more fatty acids in an oil storage organ, such as, for example, the cotton seed, wherein said plant is produced by the inventive method described hereinabove.

Such plants will exhibit a range of desirable oil characteristics which will be apparent from the preceding discussion.

In one preferred embodiment, there is provided a cotton plant having increased oleic acid and stearic acid in the seed wherein said plant is produced by sexual hybridisation between a first cotton plant having increased oleic acid in the seed compared to an isogenic non-transformed cotton plant and a second cotton plant having increased stearic acid in the seed compared to an isogenic non-transformed cotton plant, and wherein said first cotton plant and/or said second cotton plant is/are produced in accordance with the inventive method of down-regulating expression of a gene selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

In an alternative embodiment, this invention provides a cotton plant having decreased palmitic acid in the seed, wherein said plant is produced by sexual hybridisation between a first cotton plant and a second cotton plant, and wherein said first cotton plant and/or said second cotton plant is/are produced in accordance with the inventive method of down-regulating expression of a gene selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

This aspect of the invention clearly extends to cotton plants consisting of the progeny of the primary transformant plants which comprise the introduced fatty acid biosynthesis gene or gene fragment.

This aspect of the invention further extends to all plant parts, and, in particular, to seed derived from the primary transformed plant or its progeny. Preferably, the seed will comprise the introduced fatty acid biosynthesis gene or gene fragment and, more preferably, such seed will have an oil having a modified fatty acid composition in accordance with the invention (i.e. low palmitic acid and/or high oleic acid and/or high stearic acid and/or low linoleic acid).

A further aspect of the present invention extends to the oil of a cotton plant, including any transgenic cotton plants, and progeny and seed thereof, produced in accordance with the inventive method.

A further aspect of the invention extends to the gene constructs and vector molecules essentially as described herein as being capable of use in performing the inventive method. This aspect of the invention clearly extends to the use of such gene constructs to produce any plants or plant parts, such as seed, having modified oil content and/or modified fatty acid composition, or to modify oil or fatty acid content and/or composition *per se*.

The present invention is further described with reference to the following non-limiting examples.

EXAMPLE 1

General Materials and methods

Chemical reagents

All chemicals used for *in vitro* use were at least analytical grade in standard. Solutions were prepared under sterile conditions using MilliQ H_2O, and autoclaved when appropriate. All the restriction enzymes used were purchased from New England Biolabs. All the polymerase chain reactions (PCR) were performed using AmpliTaq® DNA polymerase (Perkin Elmer, USA). The origin of other enzymes, kits used are indicated together with suppliers' names in the following sections of this chapter and in the remaining chapters. The synthetic oligonucleotides were made on an Applied Biosystems (USA) Model 377 DNA synthesiser in the Division of Plant Industry, CSIRO Australia. The radionucleotide, α-^{32}P[dCTP], was supplied by Bresatec, Australia. DNA sequencing reactions were made using ABI^{PRISM} kits (Perkin-Elmer), and sequence gel electrophoresis was carried out using an ABI373 DNA sequencer.

Growth of plants

The seeds of four Australian native *Gossypium* species, including *G. australe, G. bickii, G. robinsonii and G. sturtianum* were purchased from Nindethana Seed Service, Western Australia, Australia. Seeds of other species were kindly provided by Australian Cotton Research Institute at Narrabri, NSW, Australia.

Several plants of *G. hirsutum* cv Siokra were grown in a glasshouse, using a temperature of 28/15-17°C (day/night), and natural daylight supplemented with fluorescent lighting to provide a photoperiod of 16 hours per day. Potting soils were prepared by the plant growth facility at the Division of Plant Industry, CSIRO Australia. Unless otherwise stated, all the cotton plants were grown on a soil mix consisting of 75% Pryor's mix and 25% Potting mix. Flowers were tagged at anthesis, and embryos were harvested at 5 day intervals after anthesis, until the seeds were physiologically-mature.

Recombinant DNA techniques

Unless otherwise specified, recombinant DNA techniques were carried out as described by Sambrook *et al.* (1989).

Growth of *Escherichia coli*

Cultures of *E. coli* bacteria were grown overnight at 37°C, using solid or liquid Luria Broth media (LB; 10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, 5 g/l NaCl, pH7.0). Each liquid culture was inoculated using a single bacterial colony and grown on a rotator. The appropriate antibiotics, either Ampicillin (100 μg/ml) or Kanamycin (50 μg/ml) were added to bacterial growth media at the final concentration of 100 μg/ml and 50 μg/ml, respectively.

Transformation of competent cells with plasmids

Transformation of competent cells with plasmids was performed according to the recommendations supplied with the Gene-Pulser (BioRad). Competent cells (50 μl) were combined with 5 μl deionised H_2O (dH_2O) containing 5 ng plasmid DNA or 60 ng DNA from a ligation reaction. This mixture was transferred to a pre-chilled disposable Gene-Pulser®/E.coli Pulser cuvette, 0.2 cm (BioRad), and subject to electroporation using a Gene-Pulser (BioRad), set at 1.8 kV, 125 μFD and 200 w. Immediately following electroporation, the cells were mixed with 600 μl LB broth without antibiotic, transferred into a new Eppendorf tube and incubated at 37°C for half a hour. 100 μl of culture were then plated onto a plate of solid LB media supplemented with appropriate antibiotic, and grown at 37°C overnight.

A single bacterial colony was transferred into 2 ml of LB medium containing the appropriate antibiotic in a loosely capped 15-ml tube. The culture was incubated overnight at 37°C with vigorous shaking. 1.5 ml of the culture was poured into an Eppendorf tube and centrifuged at 12,000 x g for 1 min at RT in a microfuge. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. 200 μl lysis buffer (0.1M NaOH and 0.5% SDS) was added and mixed by pipetting up and down. 200 μl 3M sodium acetate pH 6.0 was added and mixed by inverting many times and incubated on ice for 5 min. Following centrifugation for 10 min at room temperature, the supernatant was transferred into a new tube. Nucleic acids was precipitated by adding 1 ml cold ethanol, vortex to mix and centrifuge for 10 min at 4°C. The pellet was washed in 70% ethanol, centrifuging for 5 min at room temperature. After a briefly drying in a speedyvac, the pellet was resuspended in 30 μl dH_2O.

Preparation of plasmid DNA

Plasmid DNA was prepared by a modification of the alkaline lysis procedure described by Sambrook *et al.*(1989), wherein bacteria were grown in Terrific Broth (TB), instead of LB, to produce a four- to eight-fold increase in the number of bacteria per millilitre of media, which leads, in turn, to higher plasmid yield. One

litre of TB medium was made by mixing 100 ml of a sterile solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 and 900 ml of sterile, cool solution containing 12 g Bacto-tryptone, 24 g Bacto-yeast extract and 4 ml glycerol. In this modified protocol, plasmid DNA was precipitated using PEG_{8000} (polyethylene glycol), to yield high-quality, super-coiled plasmid DNA that is relatively free of contaminating chromosomal DNA and RNA.

Briefly, a single colony was transferred to 2 ml of TB medium and incubated overnight at 37°C, with an appropriate amount of antibiotic, in 50-ml polypropylene tubes. 1.5 ml aliquots of culture pelleted by centrifugation for 1 min in a microcentrifuge. The supernatant was removed and the bacterial pellet was resuspended in 200 μl of GTE buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM Na_2EDTA, pH 8.0) by pipetting up and down. 300 μl of freshly prepared lysis buffer (0.2N NaOH and 0.1% SDS) were added and mixed by inversion and incubated on ice for 5 min. This solution was neutralised by adding 300 μl of 3.0 M potassium acetate (pH4.8) and mixed by inverting the tube, and incubated on ice for 5 min. The cellular debris was removed by centrifugation for 10 min at room temperature, and the supernatant was transferred to a clean tube. RNaseA (Sigma, USA) was added to a final concentration of 20 μg/ml and incubated at 37°C for 20 min. The supernatant was extracted twice with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The plasmid DNA was precipitated by adding an equal volume of 100% isopropanol and pelleted by centrifugation for 10 min at room temperature. The DNA pellet was washed with 700 μl of 70% ethanol and then dried under vacuum. The pellet was dissolved in 32 μl of dH_2O, and then precipitated by adding 8.0 μl of 4 M NaCl and 40 μl of autoclaved 13% PEG_{8000}. After the thorough mixing, the sample was incubated on ice for 20 min, and then the plasmid DNA was pelleted by centrifugation for 15 min at 4°C. The supernatant was carefully removed and the pellet was rinsed with 700 μl of 70% ethanol. The DNA pellet was dried as before, and then resuspended in 20 μl of dH_2O.

Isolation of plant genomic DNA

All genomic DNA samples used were prepared according to Paterson *et al.* (1993). Briefly, three grams of leaf tissue were ground in liquid nitrogen to fine powder and kept in -20°C freezer until required. The powder was transferred to 20 ml of cold nuclear buffer [1.0 M glucose, 100 mM Tris-HCl (pH 7.5), 5 mM Na_2EDTA, 2% (w/v) polyvinylpolypyrrolidone (average M.W. 40,000), 0.1% diethydithiocarbamic acid, 0.1% ascorbic acid, 0.2% mercaptoethanol] and inverted to homogenise. Following centrifugation at 1000x g for 20 min at 4°C, the pellets were suspended in 10 ml of 65°C lysis buffer [100 mM Tris-HCl (pH8.0), 1.4 M NaCl, 20 mM Na_2EDTA, 2% CTAB (hexadecyl triammonium bromide), 2%polyvinylpolypyrrolidone (average M.W. 40,000), 0.1% diethydithiocarbamic acid, 0.1% ascorbic acid, 0.2% mercaptoethanol] and incubated for 20 min in 65°C water bath. An equal volume of chloroform/isoamyl alcohol was added and mixed by inverting many times. Following centrifugation at 4,500x g for 10 min, the upper aqueous phase was transferred to a new tube. This extraction step was repeated once more prior to the precipitation of DNA by addition of two volumes of 100% ethanol. The DNA was hooked out with a glass hooker and rinsed in 70% ethanol, air dried and resuspended in 500 μl of TE buffer, and then extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol. The DNA was precipitated by adding 1/10 volume of 3M NaAcetate, and two volume of 100% ethanol. Following rinsing in 70% ethanol, the DNA was air dried and resuspended in 1 ml of 1M CsCl in TE buffer.

Purification of Genomic DNA by CsCl Gradient

To purify cotton genomic DNA using cesium chloride gradients, 2.5 ml of 5.7 M CsCl in TE was added to a Beckman polyallomer 13x51 mm tube (non sealing) using a disposable pipette. 30 μl ethidium bromide (i.e. EtBr;10 mg/ml) was added to the plant genomic DNA in 1.0 M CsCl-TE buffer and laid on the top of the CsCl cushion. Following centrifugation at 35,000x rpm for 16 hours at 18°C in a SW50.1 rotor, the DNA band was removed using a 1-ml syringe and 18 gauge needle and added to a new 10-ml tube containing 5 ml of CsCl-saturated isopropanol. After

gentle mixing, the top layer containing EtBr was discarded. Three more extractions with 1 ml of CsCl-saturated isopropanol were carried out. DNA was hooked out with a glass rod following adding three volume of 70% ethanol. After resuspending the DNA in 500 μl of TE, the DNA was precipitated by adding 50 μl of 3 M NaAcetate and 1 ml of 100% ethanol and rinsed in 70% ethanol prior to resuspension in 300 μl TE buffer.

Isolation of RNA

Sterile, disposable plasticware which is essentially free of RNases was used for the preparation and storage of RNA without pretreatment. Glassware used for RNA isolation were baked at 180°C overnight. All the solutions were treated with 0.1% DEPC (diethyl pyrocarbonate) for at least 12 hours at 37°C and then autoclaved for 20 min at 15 psi on liquid cycle.

Two grams of cotton embryos or leaf tissues were ground in liquid nitrogen and mortared to a fine powder which was subsequently prickled into a beaker containing 22 mls cold extraction buffer and stirred constantly. The extraction buffer consists of 200 mM Tris-HCl pH8.5, 1.5% Lithium dodecylsulfate, 300 mM LiCl, 10 mM Na_2EDTA, 1% sodium deoxycholate, 1% Nonidet P-40. This was followed by adding 5% insoluble PVP, 90 mM mercaptoethanol, 10 mM DTT (dithiothreitol), 0.1% DEPC and stirred for 10 min prior to being transferred to a Corex tube. Then 18.4 ml of 3M ammonium acetate was added and mixed well. It was centrifuged at 6,000x rpm for 20 min at 4°C. The supernatant was transferred to a new tube and precipitated by adding 1/10 volume of 3 M NaAc, pH5.2 and 1/2 final volume of cold isopropanol and stored at -20°C for 1 hour prior to centrifugation at 6,000x rpm for 30 min using a swing rotor. The pellet was resuspended in 1 ml dH_2O and transferred to two Eppendorf tubes (500 μl in each tube). The suspension was extracted with an equal volume of phenol/ chloroform/isoamyl alcohol solution (25:24:1) and the phases were separated by centrifugation for 5 min at 4°C. The aqueous top layer was carefully transferred into a new Eppendorf tube and it was extracted again with chloroform as above.

Half volume of 5M LiCl was added to the aqueous sample, mixed well and left on ice for 3 hours prior to centrifugation at 12,000x rpm for 15 min at 4°C. The pellet was resuspended in 50 μl dH_2O. Finally, the RNA sample was precipitated by adding 5 μl NaAc and 138 μl cold ethanol and incubated on dry ice for 30 minute prior to centrifugation for 15 min at 4°C. The RNA pellet was dried in a speedyvac and then dissolved in 30 μl RNase-free H_2O.

Electrophoresis of DNA on agarose gels

Agarose mini-gels were cast from 50 ml of 1% (w/v) melted agarose solution containing 1x TAE buffer (0.04 Tris-acetate, 1.0 mM Na_2EDTA, pH 8.0), using a 5 x 8 cm mould, and a well-comb suitable for making wells of 15 μl volume. DNA samples were mixed with 0.2 volumes of 6x FLB loading buffer (15% ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol) and loaded into the wells. A DNA size marker consisting of *Eco*RI-digested SPP-1 DNA (Bresatec, Australia) was loaded into a well alongside the sample DNAs. Electrophoresis was performed at 50-70 V in 1 x TAE buffer until the dyes had separated sufficiently, after which time gels were soaked in 0.5 mg/l solution of ethidium bromide (EtBr) for 10 min, destained by rinsing in water for 10 in and photographed using UV light of wavelength 302 nm.

Transfer of cotton genomic DNA to nylon membranes for hybridisation

Cotton genomic DNA (approximately 10.0 μg DNA in 5.0 μL volume) was digested at 37°C overnight in a 20-μl reaction containing 1x concentration of an appropriate buffer, generally supplied with the restriction enzyme. A 0.7% agarose gel in 1x TBE (0.045 M Tris-borate, 1.0 mM Na_2EDTA, pH8.0) was cast using a 15x 20 cm mould. Genomic DNA digests were electrophoresed on the agarose gel overnight at 20 mA in 1x TBE buffer, until the bromophenol blue from the loading dye had run 3/4 of the length of the gel. Each gel was shaken gently in 500 ml of 0.1 M HCl for 5 min, followed by rinsing and shaken in dH_2O for a further 5 min to remove the acid. DNA was transferred from the gel to Hybond N^+ membrane (Amersham) by Southern transfer (Southern, 1975) for 4 hours, using 0.4 N NaOH as the transfer

solution. After transferring, the membrane was rinsed in 2x SSC (1.75% NaCl, 0.88% sodium citrate, pH7.0) briefly and blotted dry between 2 sheets of 3M membrane paper at room temperature.

Transfer of cotton RNA to nylon membranes for hybridisation

To prepare a 300 ml of 1% agarose gel for RNA electrophoresis, 3 grams agarose and 30 ml 10x MOPS [3-(N-morpholino)propanesulfonic acid] buffer were added to 255 ml dH_2O and dissolved by heating. Formaldehyde (16.2 ml of 37% stock solution) was added to the agarose solution when it had cooled to 50°C, and mixed by swirling. Total RNA (20 μg) was mixed with 20 μl of loading buffer and then heated to 95°C for 2 min to denature RNA. The loading buffer was freshly prepared by mixing 0.72 ml formamide, 0.16 ml 10x MOPS buffer, 0.26 ml formaldehyde (37%stock), 0.1 ml 80% (v/v) glycerol, 0.08 ml bromophenol blue (saturated solution) and 0.18 ml dH_2O. Ethidium bromide (1 μl) was then added to each sample. The RNA samples were electrophoresed in 1x MOPS buffer until the bromophenol blue dye migrated three-fourths along the length of the gel. The RNA was viewed using under UV light using a transilluminator, and photographed. The two abundant rRNA species visible under UV light, namely the 28S rRNA (5 kb) and 18S rRNA (2 kb), were used as molecular weight standards. Immediately prior to transfer, the RNA gel was rinsed for 20 min each in two changes of 500 ml of 10x SSC to remove formaldehyde from gel. This was immediately followed by transferring the RNA to Hybond N^+ membrane using 0.05 N NaOH as the transfer buffer, for a period of 3 hours.

Preparation of ^{32}P-labelled DNA probes

Radioactively labelled probes were synthesised by random priming, essentially as described by Feinberg and Vogelstein (1983), using a random priming kit obtained from Amersham (USA). Purified cloned insert DNA (20 ng) was combined with 6.0 μl of random oligonucleotides (Amersham). This the mixture was incubated at 95°C for 5 min to denature the DNA and quickly chilled on ice for 5 mins. Reactions were carried out at 37°C, for 1 hour, in a total volume 25 μl, comprising

10 μl of probe labelling buffer (0.5 M HEPES, 0.125 M Tris-HCl, 12.5 mM DTT, 12.0 mM $MgCl_2$, 1.0 mg/ml BSA); 2.5 μl dNTP mixture (0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP); 1.0 unit Klenow fragment; 3.0 μl [α-^{32}P]dCTP (10 μCi/μl), and MilliQ H_20. To remove the unincorporated dNTPs, the probe was purified by passing the reaction mixture through a Sephadex® G-50 NICK® Column (Pharmacia) according to the manufacturer's instructions.

Hybridisation and autoradiography

Nylon membranes with nucleic acid bound thereto were prehybridised in hybridisation bottles containing 5-20 ml of the prehybridisation solution essentially as described by Khandjian (1987) (50 mM Tris-HCl pH7.5, 1M NaCl, 50% formamide, 10x Denhardt's solution, 10% dextran sulfate, 1% SDS, 0.1% sodium pyrophosphate, 0.1 mg/ml herring sperm DNA). The bottles were placed in a hybridisation oven equipped with a rolling apparatus for at least 4 hours at 42°C.

Following prehybridisation, labelled probe DNA was denatured by heating for 10 min at 95°C, and then placed on ice for 5 min. Denatured probed was added to the prehybridisation mixture. The hybridisation was performed at 42°C for 16 hours. Unless otherwise stated, membranes were then briefly washed in 2x SSC, 0.1% SDS at 65°C. This was followed by two further washes in 0.2x SSC, 0.1% SDS at 65°C for 15 min each. Autoradiography was performed for one to five days at -80°C with a Kodak X-ray film and an intensifying screen.

Construction of a cottonseed cDNA library

Cotton poly(A)$^+$ RNA was isolated from total RNA prepared as described above, using a mRNA purification kit (Pharmacia), essentially as described by the manufacturer. To prepare cDNA, a cDNA synthesis kit (Pharmacia) was used, essentially as described by the manufacturer, using 1-5 μg poly(A)$^+$ RNA as starting material. The double-stranded cDNA product was blunt-ended, and ligated to EcoRI/NotI adaptors, using standard procedures. Following the removal of

excess unligated adaptors, the cDNA was cloned into the bacteriophage vector Lambda ZAPII (Stratagene, USA), and packaged using a commercially-available packaging system (Stratagene, USA), according to the manufacturer's instructions.

Titration of bacteriophage particles in cDNA libraries

To prepare host cells, a single colony of the bacterial host XL1-Blue MRF' was transferred into 50 ml of LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$ in a sterile Erlenmeyer flask and grown overnight with shaking at 30°C. This lower temperature ensures that the cells will not overgrow. The cells were centrifuged in a sterile conical tube for 10 min at 2,000x rpm. The pellet was gently resuspended in 10 mM $MgSO_4$ to the final cell density of OD_{600}=0.5.

To titre the bacteriophage, each of a serial dilution of the packaging reaction (1, 1/10, 1/100), and 200 μl of the host cells, were mixed and incubated at 37°C for 15 min. Then, 2-3 ml of cooled (48°C) top agar (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 1% NZ Amine, pH7.5, 0.7% agarose), 15 μl of 0.5 M IPTG (isopropylthio-β-Δ-galactoside) and 50 μl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 250 mg/ml in dimethylformamide) was added, and poured onto NZY plates (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 1% NZ Amine, pH7.5, 1.5% agar). Plates were incubated for at least 6-8 hours at 37°C.

The cDNA libraries described herein generally contained about 92% recombinant bacteriophage particles, in a total of about 1.5x 10^7 plaque forming units (pfu) per ml of unamplified library.

Amplification of cDNA libraries

Twenty aliquots of the packaged unamplified cDNA library, each containing about 5x 10^4 pfu, were mixed with 600 μl of host cells prepared as described hereinabove, in Falcon 2059 tubes, and the mixtures were incubated at 37°C for

15 min. Each aliquot of infected bacteria was mixed with 6.5 ml of cooled (about 48°C) melted top agar and spread evenly onto a freshly poured 150-mm plate of bottom agar. The plates were incubated at 37°C for 6-8 hours. Each of these plates was then overlaid with 8-10 ml of SM buffer and incubated at 4°C overnight with gentle rocking to elute the bacteriophage. The bacteriophage suspension was recovered from each plate and pooled into a sterile polypropylene container and chloroform was added to a final concentration of 5%(v/v). After 15 min incubation at room temperature, cell debris was removed from the bacteriophage suspension by centrifugation at 2,000x g for 10 min. The supernatant was recovered and aliquots were stored in 7% DMSO (dimethyl sulfoxide) at -70°C.

The titre of amplified cottonseed cDNA libraries prepared according to this procedure was generally about 2x 10^9 pfu/ml.

Screening of cDNA libraries

Amplified cottonseed cDNA libraries were plated onto 20 petri dishes (150 mm) containing NZY medium, by infecting 600 μl host cells prepared as described previously (*E. coli* XL1-Blue MRF' cells) with about 50,000 pfu, and then adding 6.5 ml top agar to each plate as described above. When the plaques became visible, generally after about 8 hours of incubation at 37°C, the plates were chilled at 4°C for about 2 hours.

Plates were overlaid with Hybond N^+ (Amersham) membranes which had been numbered previously, and the membrane's position on each plate was keyed. When the membranes had been thoroughly adsorbed to the plates, they were removed gently so as to not remove the top agar, and placed onto several layers of 3MM membranes saturated with denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 7 min. The membranes were then neutralised, by soaking on two changes of neutralising buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH7.0, 0.001 M EDTA) for 3 min each. The membranes were washed in 5x SSC briefly and blotted dry between two layers of 3 MM membranes. Membranes were then fixed by laying on top of

several layers of 3 MM membranes saturated with 0.4 N NaOH for 10 min and rinsed in two changes of 5x SSC briefly before being blotted dry between 3MM membranes.

For hybridisations, the membranes were soaked in 2x SSC briefly, transferred to enough prehybridisation buffer (described hereinabove) to cover the membranes completely, and incubated at 42°C for at least 4 hours with gentle shaking. Following prehybridisation, the [α-^{32}P]dCTP-labelled DNA probe (described hereinabove) was denatured by incubating at 95°C water bath for 5 min, chilled rapidly on ice, added to the prehybridisation buffer, and the reaction incubated at 42°C for 16 hours.

The hybridisation solution was removed and the membranes were immediately washed in 2x SSC, 0.1% SDS briefly at 65°C, unless otherwise stated. This was followed by two consecutive washes in 0.2x SSC, 0.1% SDS for 15 min each. Unless otherwise stated, all the washes were normally carried out at 65°C. Following this washing, the excess liquid was removed by blotting on Whatman 3MM paper. The membranes were then placed between two sheets of plastic wrap in a cassette and exposed to X-ray film (Kodak) overnight at -80°C with an intensifying screen. The positive-hybridising plaques were identified and transferred from the original plates, into 1 ml of SM buffer and 20 μl of chloroform and mixed.

Positive-hybridising plaques were purified by re-screening as before, using 50-100 plaques per small NZY plate.

Following plaque-purification, the ExAssist/SOLR system (Stratagene, USA) was used to excise the pBluescript SK(-) phagemid from the Lambda ZAPII vector, as described by the manufacturer.

Nucleotide sequence analysis

DNA sequencing was performed with an ABI 373 system (Applied Biosystems) using dye terminator and dye primer sequencing reactions. Sequence comparisons and alignments were performed with programs in the GCG package (Devereux *et al.*, 1984).

EXAMPLE 2

Determining oil content and fatty acid composition of developing and mature cotton seed

The total lipid content of cottonseed, or seed oil, was extracted by a modification of the method of Folch *et al.* (1957). Dry cottonseeds were ground in a coffee grinder. Immature embryos were harvested immediately before grinding into powder in liquid nitrogen with pestles and mortars. Approximately 4 grams ground powder from mature seed samples were weighed out and the exact weights were recorded. Because of the limited availability of immature embryos, only 1-2 gram samples were used. They were placed into an Erlenmeyer flask. Total lipids were then extracted with 60 ml of Folch reagent (2:1 v/v chloroform:methanol solution, 15 ml per gram of sample). The extracts were filtered through Whatman No. 1 filter paper into a 100-ml graduated cylinder and made up to 60 ml volume. Then they were washed with 12 ml of saline solution (0.88% NaCl, 20% volume of the extract) and the phases were allowed to separate. The upper phase, containing water, methanol and water soluble material was siphoned off and discarded. The lower phase of chloroform and lipids was further washed with a chloroform:methanol:saline (3:47:48 v/v) solution. Again phases were allowed to separate. The final volume of the lower chloroform:lipid was recorded and the upper layer siphoned off and discarded. 10 ml of the chloroform extract and 1 ml of internal standard, heptadecanoic acid (C17:0, 5.196 mg/ml) were transferred into a preweighed culture tube with a screw cap. The sample was evaporated to dryness under a nitrogen gas stream on a 70°C heating block. The amount of crude lipids

per gram of sample was determined by weighing the tube containing the dried sample.

Five millilitres of the methylating reagent, a mixture of boron trifluoride:methanol :hexane (35:45:20) was added to the culture tube containing dried lipid and capped tightly with a teflon lined crew cap. Then it was incubated in a 90°C water bath for 45 min with frequent shaking. After cooling down to room temperature, 5 ml of water and 5 ml of hexane were added to the mixture. Following shaking and phase separation, the top hexane layer containing the fatty acid methyl esters was transferred into a new tube and used in the GLC analysis.

The fatty acid composition of the lipids was determined by GLC, using a Varian Gas Liquid Chromatography apparatus (Model 3400), equipped with a fused capillary column DB624 (J & W Scientific). The injector and detector temperature were 220°C and 240°C, respectively. The nitrogen flow rate was 30 ml/minute. The following program was used in the GLC analysis:

(i) the column temperature was held at 70°C for 3 min;

(ii) the column temperature was increased to 160°C at a rate of 30°C/min, and then held at 160°C for 10 min; and

(ii) the column temperature was increased to 230°C at a rate of 7°C/min, and then held at 230°C for 4 min.

A fatty acid standard containing lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid(C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), behenic acid (C22:0), eicosadienoic acid (C20:2), and lignoceric acid (C24:0), was used to identify the fatty acids present in cotton seed oil.

Fatty acid peaks were identified by comparing the fatty acid methyl ester peaks and retention times of standards with sample peaks. An electronic integrator was

used to calculate the total area of peaks and the area of each fatty acid peak was expressed as a percentage of the total area.

EXAMPLE 3

Oil content and fatty acid composition of cotton seed derived from several elite cotton cultivars

Using the procedure described in Example 2, we determined the oil composition of cotton seed derived from several elite Australian cotton cultivars, and some other commercial and non-commercial cotton species.

In all cultivars tested, palmitic acid (C16:0) represents the major saturated fatty acid, while low level of other saturated fatty acids including myristic acid (C14:0) and stearic acid (C18:0) were also detected. Oleic acid and linoleic acid were the two major unsaturated fatty acids,whilst a very low level of palmitoleic acid (C16:1) was also detectable. The low level of stearic acid in cottonseed oil is probably because of the strong activity of a Δ9 stearoyl-ACP desaturase enzyme which converts most of the stearic acid to oleic acid, which is then further desaturated by a microsomal ω-6 desaturase, to form linoleic acid. Linoleic acid accounts for more than 50% of total fatty acid composition of cotton seed, indicating a strong microsomal ω-6 desaturase activity in cottonseeds.

GLC analyses did not differentiate cyclopropenoid fatty acids (CPFA) from other unsaturated fatty acids. Previous studies have demonstrated that cottonseed oil contains less than 1% cyclopropenoid fatty acids (CPFA), mainly sterculic and malvalic acids, which contain one double bond at the site of a propene ring, either at the 9-10 or 8-9 position (Phelps *et al.*, 1964; Allan *et al.*, 1967). CPFA is unstable during the standard lipid extraction process, but derivatives of CPFA have been quantitated by modified GLC technique and high performance liquid

chromatography (Fisher and Schuller, 1981; Fisher and Cherry, 1983; Pandey and Subrahmanyam, 1988).

The mean values for percentage oil content and the component fatty acids in the cottonseed oils from 20 entries are reported in Table 4. Student-Newman-Keuls test for the oil content and component fatty acids of various entries indicated that species or genotypes have a significant influence on the oil content and the majority fatty acid compositions of cottonseed oil (except for C14:0) at the 5 percent level of probability.

The four Australian wild diploid *Gossypium* species contained significantly lower oil content than the remaining entries examined. They were also found to contain a significantly higher level of linoleic acid, and lower levels of palmitic and oleic acids. In contrast, the only A-genome diploid species tested, *G. arboreum*, was found to contain significantly high levels of oleic acid, however this species also had significantly low linoleic acid. This result is largely in agreement with previous studies on the chemical survey in genus *Gossypium* (El-Nockrashy *et al.*, 1969; Khattab *et al.*, 1977).

The major Australian elite cotton cultivars, SicalaV2, SiokraL22, SiokraL23 and CS50 had the highest oil content, whereas Sicala33 and Sicala34 were found to contain moderate levels of oil which were not significantly different from the American cultivar, Acala R.

With respect to the fatty acid composition of the Australian elite cotton cultivars, SiokraL22, SiokraL23, Sicala33, and Sicala34, contain relatively high levels of palmitic acid and moderate levels of unsaturated fatty acids.

The present study indicates that there are some, but limited variations of oil content and fatty acid composition among the elite Australian cotton varieties.

EXAMPLE 4

Developmental changes in the fatty acid composition of cotton seed

The oil accumulation and changes of fatty acid composition in developing cottonseed embryos aged 15-65 days-after-anthesis (DAA) is illustrated in Figure 1. In particular, little oil was detected in seeds at 15 DAA. Between 15-20 DAA, oil accumulation was also relatively low, however a rapid increase in oil accumulation occurred between 20-35 DAA. After 35 DAA, the rate of oil accumulation in the seed slows, and is essentially constant. These observations are consistent with previous reports (El-Nockrashy *et al.*, 1975; Kajimoto *et al.*, 1979).

We also measured the amounts of the six major fatty acids present in cotton seed, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2), in developing embryos (Figure 1). The rate of synthesis of linoleic acid was higher than the rates of syntheses for the other fatty acids. A rapid increase in synthesis of linoleic acid was observed in the period 15 to 30 DAA, concomitant with the rapid increase of percent oil content, wherein the linoleic acid content rose from 22% to almost 50%. There was only a slight increase thereafter. We also observed a continuous decrease in the percentage of palmitic acid in the seed, between 15-35 DAA, a decrease which slowed in later developmental stages. Interestingly, oleic acid levels decreased gradually throughout development of the cottonseed. Stearic acid content of the seed decreased from about 9% of total lipids at 15 DAA, to only about 2.5% at 30 DAA, and remained at that level thereafter. Myristic acid and palmitoleic acid remained at relatively low levels throughout the period of embryo development.

TABLE 4

Oil content and fatty acid composition of seed oil of some *G. hirsutum* cotton cultivars and other *Gossypium* species

Entries	Oil Content	Fatty Acid (%)					
	(%)	14:0	16:0	16:1	18:0	18:1	18:2
G. australe	16.2b	0.7a	21.5a	1.6b	1.7a	11.0b	62.1f
G. bickii	14.6a	0.8a	20.6a	0.9a	3.5c	8.6a	64.1g
G. robinsonii	17.5b	0.9a	22.8abc	0.6a	2.7b	10.4ab	60.0e
G. sturtianum	17.3b	1.1a	20.4a	0.5a	2.6b	11.2b	62.5fg
G. arboreum	22.4de	0.7a	21.0a	1.0a	3.6c	27.2e	45.3a
G. barbadense	21.1cd	0.7a	24.4bcd	0.7a	2.2ab	19.4d	52.0bc
G. hirsutum:-							
cv Acala R	23.0ef	0.6a	24.4bcd	0.5a	2.3ab	16.9cd	54.4cd
cv BAR7/8	21.1cd	0.9a	23.9bc	0.6a	2.3ab	15.6c	56.1d
cv DP90	22.0cde	0.7a	26.9d	0.6a	2.3ab	16.3cd	52.5bc
cv MCU-5	20.6c	0.7a	22.4ab	0.8a	2.1ab	16.4cd	56.1d
cv Lintless 28	23.7efg	0.8a	24.3bc	0.5a	2.6b	18.7cd	52.6bc
cv Lintless 53	23.7efg	0.8a	25.5cd	0.5a	3.7c	18.3cd	50.6b
cv Sicala33	23.2ef	0.8a	24.6bcd	0.7a	2.7b	16.7cd	53.4c
cv Sicala34	23.0ef	0.7a	24.6bcd	0.5a	2.5b	16.7cd	54.0cd
cv CS50	25.0gh	0.6a	24.1bc	0.4a	2.5ab	17.7cd	54.1cd
cv CS65	23.8efg	0.9a	22.6ab	0.6a	2.5ab	16.3cd	56.4d
cv Siokra L22	24.5fgh	0.8a	24.9bcd	0.7a	2.7b	17.2cd	53.1bc
cv Siokra L23	24.7fgh	0.7a	25.1bcd	0.7a	2.5ab	17.5cd	52.7bc
cv Siokra 1-4	25.5h	0.7a	24.4bcd	0.5a	2.4ab	17.5cd	54.2cd
cv Sicala V-2	26.0h	0.7a	23.8bc	0.5a	2.4ab	17.9cd	54.1cd

* The least significant differences are calculated for the 5% level of probability. Within columns means having a letter in common are not significantly different.

The data presented in Figure 1 suggest that the oil content and fatty acid composition of cotton seed is determined at an early stage of development, approximately 20-35 DAA. In fact, the majority of the total oil found in the mature

cottonseed is synthesised during latter 30 days of embryo development, and this appears to be a critical time in cotton seed oil production.

EXAMPLE 5

Isolation and cloning of cDNAs encoding cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase)

A nucleotide sequence comprising a cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene was isolated essentially as described by Liu, Q., *et al.* (1996).

Briefly, a pair of degenerate PCR primers were synthesised that contained some possible codons corresponding to the open reading frames (ORFs) of peptide sequences among Δ9- stearoyl-ACP desaturase cDNAs from castor bean (Shanklin and Somerville, 1991), cucumber (Shanklin *et al.*, 1991) and flax (Singh *et al.*, 1994). The sequences of the primers are as follows (degenerate positions are in parentheses):

D9S (SEQ ID NO: 8): 5'- ATGGC-(G/T)CT(C/G)A(A/G)GCT(C/G/T)CAT(C/G)C -3'; and

D9A (SEQ ID NO: 9): 5'- TCA(G/C)AG(C/T)TT(C/A)AC(T/C)TG-(T/C)CTAT -3'.

Total RNA was isolated from cottonseed embryos at 25-35 days post anthesis, and poly(A)$^+$ RNA purified therefrom according to procedures described herein above.

Single stranded cDNA template for reverse transcriptase-PCR (RT-PCR) was synthesised at 45°C from poly(A)$^+$ RNA using a Superscript kit (Gibco BRL),

according to the manufacturer's instructions. RT-PCR amplifications were carried out in 50-μl reaction volume, containing 50 pmol of each primer D9S and D9A, and single-stranded cDNA derived from 20 ng of poly(A)$^+$ RNA. The mixture was heated to 96°C for 5 min prior to commencing the reaction, and 3 units of AmpliTaq® DNA polymerase (Perkin-Elmer Cetus) was added. Cycling of reaction mixtures was performed in a Corbett FTS960 thermal cycler, using 40 cycles of the following program: (i)94°C for 30 sec;

(ii)48°C for 30 sec; and

(iii)72°C 1 min 10 sec.

These 40 cycles were followed by incubation for 10 min at 72°C.

Under these conditions, an approximately 1 kb DNA fragment comprising a protein-encoding region, was obtained. The identity of the cloned PCR product as a partial cDNA encoding Δ9- stearoyl-ACP desaturase, was confirmed by DNA sequencing. This fragment was cloned into a T®-vector (Promega).

The partial cDNA was excised from purified plasmid DNA, and radio-labelled with [α-^{32}P]dCTP as described hereinabove. The labelled probe was used to screen a cottonseed cDNA library,prepared using the λZAPII vector (Stratagene) and poly(A)$^+$ RNA isolated from the embryo tissues of developing seed of *G. hirsutum* cv Deltapine-16 as described hereinabove, under high stringency hybridisation conditions. Ten plaques which showed strong positive signals in the primary screening were selected and further purified by secondary and tertiary screenings. Phagemid containing the Δ9- stearoyl-ACP desaturase cDNA was excised *in vivo* using ExAssist/SOLR system.

All of the 10 clones idenified were identical, based on the partial nucleotide sequence and restriction mapping analyses. One clone, designated ghSAD-1, was

completely sequenced (SEQ ID NO: 1; Figure 2). The nucleotide sequence of ghSAD-1 is also publicly available under the GenBank Accession No. X95988.

Clone GhSAD-1 is 1553 bp in length, and comprises a 12 bp 5'-UTR, a 350 bp 3'-UTR, and a 1191 bp ORF which encodes a polypeptide of 397 amino acid residues in length (SEQ ID NO: 2). The nucleotide sequence surrounding the putative ATG start site has 78% identity to the consensus for plant translation initiation proposed by Lütcke *et al.* (1987). The identity of ghSAD-1 as encoding Δ9 stearoyl-ACP desaturase was confirmed by analysis of its sequence identity to other Δ9 stearoyl-ACP desaturase-encoding cDNAs in the GenBank database.

The deduced *G. hirsutum* Δ9 stearoyl-ACP desaturase polypeptide contains an apparent plastid transit peptide sequence at its N-terminus, consistent with its plastid localisation. Based on homology with the castor bean polypeptide (Knutzon *et al.*, 1991), the amino acid residue at position 32 of the polypeptide encoded by ghSAD-1 is the most likely cleavage site for the plastid transit peptide. Residues immediately preceding amino acid 33 of SEQ ID NO: 2 conform closely to the consensus cleavage site for plastid transit peptides proposed by Gavel and von Heijne (1990). Accordingly, it is possible that the ghSAD-1 cDNA encodes a precursor polypeptide comprising a 32 amino acid-long transit peptide, to facilitate translocatation of the polypeptide into the proplastid. Subsequent cleavage of the transit peptide possibly produces a mature protein of 365 amino acid residues in length. As with other, previously characterised, plant Δ9 stearoyl-ACP desaturases, the deduced amino acid sequence of ghSAD-1 is highly hydrophilic, consistent with the soluble nature of the enzyme. No transmembrane helices were found in the whole polypeptide sequence using the internet program SOSUI (http://www.tuat.ac.jp/~mitaku /sosui/).

As indicated in Table 5, there is considerable homology between ghSAD-1 and Δ9 stearoyl-ACP desaturase cDNAs from other plant species at the nucleotide and

amino acid levels. As expected, the mature Δ9 stearoyl-ACP desaturase proteins are more highly conserved, than the transit peptides of the full-length polypeptides.

Amino acids 322-342 of SEQ ID NO: 2 (Figure 2) conform to a fatty acid desaturase family II signature (http://www.genome. ad.jp/SIT/MOTIF.html). An iron-binding motif, containing two repeats of the amino acid sequence Glu-Xaa-Xaa-His (i.e. EXXH) separated by approximately 100 amino acids, is also found in the cotton Δ9 stearoyl-ACP desaturase polypeptide (bold type in Figure 2), as with other class II di-iron proteins, including the R2 component of a ribonucleotide reductase and the soluble bacterial hydrocarbon hydroxylases (Fox *et al.*, 1994).

EXAMPLE 6

Expression of the ghSAD-1 gene during cotton seed development

Total RNA, from developing cotton seed embryos at 25, 30, 36, 45 DAA, or leaves, was isolated, electrophoresed on an agarose gel, trasnferred to nylon membranes, and probed with a labelled DNA fragment derived from the 3'-UTR of the ghSAD-1 clone, essentially as described hereinabove. Owing to the difficulty in obtaining RNA samples from very early stages of embryo development, gene expression prior to 25 DAA was not examined.

As demonstrated in Figure 3, high expression was detected in all four stages of embryo development examined. However, there was no detectable transcript in leaf tissue. Highest seed-specific expression was obseerved at 30 DAA, and 35 DAA. We speculate that the expression of ghSAD-1 is induced well before 25 DAA..

TABLE 5

Similarities between ghSAD-1 and other stearoyl-ACP Δ9-desaturase cDNAs

Plant Species	Accession No.	Similarity (%) to ghSAD-1 ORF (nt)	overall identity	Mature Peptide	Reference
Arabidopsis thaliana	X93461	71.4	84.9	86.3	GenBank X93461
Arachis hypogaea	AF172728				Tate *et al.*, 1999
Brassica napus	X63364	71.8	86.1	88.5	Slocombe *et al.*, 1992
Brassica juncea	AF153420				Vageeshbau *et al.*, 1999
Brassica rapa	X60978	73.0	86.9	88.8	Knutzon *et al.*, 1992
Elaeis guineensis	U68756	72.4	87.5	89.0	Shah and Rashid, 1996
Sesamum indicum	D42086	74.7	88.9	90.4	Yukawa *et al.*, 1994
Carthamus tinctorius	M61109	72.8	89.2	91.2	Thompson *et al.*, 1991
Coriandrum sativum	M93115	63.3	77.8	78.5	Cahoon *et al.*, 1992
Cucumis sativa	M59858	73.8	88.4	89.9	Shanklin *et al.*, 1991
G. hirsutum	AJ132636				Liu *et al.* 2000
G. hirsutum	AI730379				Blewitt *et al.*, 1999
Glycine max	L34346	74.9	88.8	90.4	Chen and Moon, 1995
Helianthus annuus	U70374	70.8	85.4	88.0	GenBank U70374
Linum usitatissimum	X90762	68.4	80.7	82.5	Singh *et al.*, 1994
Linum usitatissimum	AJ006957				Jain, 1998
Linum usitatissimum	AJ0069578				Jain, 1998
Asclepias syriaca	U60277	59.2	76.3	76.8	Cahoon *et al.*, 1997
Olea europaea	U58141	69.9	84.7	86.3	Baldoni *et al.*, 1996
Pelargonium xhortorum	U40344	62.7	77.9	79.1	Schultz *et al.*, 1996
Persea americana	AF116861				Madi and Pruskey, 1999
Oryza sativa	D38753	67.7	82.1	84.9	Akagi *et al.*, 1995
Ricinus communis	M59857	77.6	89.4	90.7	Shanklin *et al.*, 1991
Sesamum indicum	D42086				Yukawa et al., 2000
Solanum commersonii	X78935	71.5	87.6	87.6	GenBank X78935
Simmondsia chinensis	M83199	69.9	86.1	89.0	Sato *et al.*, 1992
Spinacia oleracea	X62898	71.0	86.2	88.2	Nishida *et al.*, 1992
Solanum tuberosum	M91238	70.8	84.8	86.6	Taylor *et al.*, 1992
T. alata pTAD2	U07597	69.6	86.2	88.5	Cahoon *et al.*, 1994
T. alata pTAD3	U07605	70.5	85.9	87.4	Cahoon *et al.*, 1994
T. alata Δ6-desaturase	U09269	65.0	75.0	77.8	Cahoon *et al.*, 1994

EXAMPLE 7

Organisation of the Δ9 Stearoyl-ACP desaturase gene family

We analysed the organisation of the cotton Δ9 stearoyl-ACP desaturase gene family by Southern blot hybridisation of cotton genomic DNA samples, using labelled DNA probes consisting of the 3'-UTR of the ghSAD-1clone, or alternatively, the entire coding region of ghSAD-1, under high stringency hybridisation conditions, essentially as described herein above.

Genomic DNAs tested were from *G. barbadense*, *G. hirsutum* (cv Deltapine-16), *G. hirsutum* (cv Siokra), *G. herbaceum, G. raimondii.* The cotton DNAs were digested with *Eco*RI, *Hin*dIII, or *Xba*I prior to electrophoresis, because these enzymes do not have sites in the region covered by the probe DNA sequences.

As shown in Figure 4-A, the ghSAD-1-specific probe consisting of the 3'-UTR of ghSAD-1 detected two strong bands in each of the allotetraploids, and a single strong band in each of the two diploids, for all restrction enzyme digests tested. These data suggest the presence of two copies of the *ghSAD-1* gene in each of the tetraploids, and only one copy in each of the diploids, consistent with the ploidy of each cotton.

In contrast, the entire ghSAD-1 coding region detected a complex pattern in cotton DNA, consisting of multiple fragments in each of the three enzyme digests (Figure 4-B). These data clearly indicate that the Δ9 stearoyl-ACP desaturase is encoded by a multigene family which is composed of 6-8 genes, or gene copies, in diploid cotton genomes, and probably twice as many in the allotetraploid cottons, *G. barbadense* and *G. hirsutum.*

Moreover, the allotetraploid cottons tested, *G. barbadense,* and two *G. hirsutum* cultivars, share highly conserved restriction fragment length patterns, with only minor differences. However, the two diploid species are distinctively different. It was estimated that the A- and D-genomes of cotton diverged from a common

ancestor 6-11 million years ago, whereas the divergence of the two tetraploids occurred much more recently, about 1-2 million years ago (Endrizzi *et al.*, 1985; Wendel and Albert, 1992). Among the currently existing diploid cottons, the A-genome diploid species *G. herbaceum,* and the D-genome diploid species *G. raimondii,* are regarded as being most-closely related to the progenitor species of the modern tetraploid cotton (Endrizzi *et al.*, 1985).

In this respect, of the two *Eco*RI fragments in each of the three tetraploids (lanes 1-3 of Figure 4-A), the lower *Eco*RI fragment appears to be the same as that in the A-genome diploid *G. herbaceum* (lane 4 of Figure 4-A), suggesting that this gene is derived from the A-genome. Similarly, the upper *Eco*RI fragment in each of the three tetraploids (lanes 1-3 of Figure 4-A) corresponds to the fragment present in the D-genome diploid *G. raimondii* (lane 5 of Figure 4-A), suggesting that this gene is derived from the D-genome. Even among the complex patterns in Figure 4-B, some hybridising fragments present in the tetraploid cottons are identical to those of the diploid species tested, suggesting an assignment of subgenomic origins of the tetraploid genes.

In summary, data presented in Figure 4 suggest that the cotton Δ9 stearoyl-ACP desaturase gene, ghSAD-1, is a member of a highly-conserved multi-gene family in tetraploid cotton, comprising at least 6-8 genes, or gene copies, per diploid genome, and possibly derived from the A and D genomes.

EXAMPLE 8

Isolation and cloning of cDNAs encoding cotton fatty acid Δ12-desaturase (oleoyl-PC Δ12 desaturase)

A nucleotide sequence comprising a cotton fatty acid Δ12-desaturase (oleoyl-PC Δ12 desaturase) gene was isolated essentially as described by Liu, Q., *et al.* (1999).

Briefly, a heterologous probe, consisting of the entire coding region of the *B. juncea* microsomal ω-6 desaturase, was used to screen the cottonseed cDNA library described hereinabove. Twelve positive-hybridising plaques were identified in a primary screen of approximately 1 x 10^6 pfu , eleven of which were purified by two further consecutive screenings, and their cDNA inserts characterised by nucleotide sequence determination, essentially as described hereinabove.

Restriction enzyme mapping, and partial DNA sequence analysis, indicated that the rescues cDNA clones harboured the same insert, designated ghFAD2-1.

Analyses of the nucleotide sequences of ghFAD2-1 revealed that it was truncated at the 5' end. To obtain the missing 5' end of ghFAD2-1, a PCR approach using crude DNA exacts from λ cDNA library as DNA template was employed. A sample of the bacteriophage containing the cotton seed cDNA library (approximately 1 x 10^9 pfu) was extracted by phenol/chloroform and chloroform, followed by ethanol precipitation. The pellet was air-dried and dissolved in 20 μl H_2O. The 5′-end of the cDNA was amplified using a cDNA-specific primer, designated Δ12A4, in combination with the forward primer (i.e. the primer which anneals to the forward priming site in the pBluescript vector), or alternatively, the reverse primer (i.e. the primer which anneals to the reverse priming site in the pBluescript vector), that bind in the region close to the 5'- and 3'- *Eco*RI cloning sites of that vector, as follows:

Primer Δ12A4 (SEQ ID NO: 10): 5'-GCATAGGTCATGGACCACGT-3';
Forward Primer (SEQ ID NO: 11):5'-GTAAAACGACGGCCAGT-3';
Reverse Primer (SEQ ID NO: 12): 5'-GGAAACAGCTATGACCATG-3'.

A 50 μl PCR reaction mixture contained 200 μM dNTP, 1x PCR buffer, 20 pmol of each of the primers, 5 μl of cDNA library extract, and 1 unit of Taq polymerase. PCR was conducted by heating to 94°C for 2 min, followed by 30 cycles, at 94°C for 45 seconds, 57°C for 1 min, and 72°C for 1 min. After the last cycle, reactions were incubated for 10 min at 72°C. The PCR product was purified by Wizard PCR

Preps DNA Purification System (Promega) and cloned into T-vector (Promega) according to manufacturer's instructions.

The nucleotide sequence of one amplified clone matched the 5'-terminal sequence of the original cDNA clone. The nucleotide sequences of the original clone and the amplification product were joined (SEQ ID NO: 3), to produce a full-length cDNA of 1411 bp in length, encoding a polypeptide of 386 amino acids in length (SEQ ID NO: 4; and Figure 5).

A second cDNA encoding a cotton microsomal ω-6 desaturase, of 1422 nucleotides in length, has also been isolated and is designated ghFAD2-2 (Accession No. Y10112). The nucleotide sequence and deduced amino acid sequence of ghFAD2-2 is presented in Figure 6 (SEQ ID NO: 5). The ghFAD2-2 cDNA clone is divergent from ghFAD2-1 in so far as it possesses unique 5'- and 3'- UTRs, indicating that these genes have evolved independently since their divergence from a common ancestral gene. Gene-specific probes, based on the unique 3'-UTR sequences of ghFAD2-1 and ghFAD2-2, have been used in Southern and Northern blot analyses, to determine gene organisation and expression, respectively.

As shown in Figure 7, the alignment of the microsomal ω-6 desaturases from cotton (ghFAD2-1 and ghFAD2-2), soybean (gmFAD2-1, FAD2-2), *A. thaliana* (atFAD2), and the microsomal ω-3 desaturase from *B. napus* (bnFAD3), and the plastid ω-6 desaturase from soybean (gmFAD6), reveals a high conservation between the microsomal ω-6 desaturases. Moreover, the seed-specific proteins of cotton (ghFAD2-1) and soybean (gmFAD2-1) are most closely-related to each other, whilst cotton ghFAD2-2 is most similar to the constitutively-expressed soybean protein, gmFAD2-2. Significantly lower sequence identity occurs between the *B. napus* microsomal w-3 desaturase prtoein, bnFAD3, and any of the microsomal ω-6 desaturases. The plastid ω-6 desaturase, gmFAD6, remains the least similar sequence to all the microsomal desaturase amino acid sequences. This analysis confirms previous studies (Yadav *et al.*, 1993).

The three histidine boxes which were shown to be highly conserved in all membrane-bound desaturases (Schmidt *et al.*, 1994), were also observed in the *G. hirsutum* microsomal ω-6 desaturases, ghFAD2-1 and ghFAD2-2. As shown in Figure 7, among the five microsomal ω-6 desaturases listed, the deduced consensus amino acid residues for the three histidine box motifs are as follows:

(i) His-Glu-(Cys/Trp)-Gly-His-His [i.e. HE(C/W)GHH] (SEQ ID NO: 26);

(ii) His-Arg-Arg-His-His (i.e. HRRHH) (SEQ ID NO: 27); and

(iii) His-Val-Ala-His-His (i.e. HVAHH) (SEQ ID NO: 28).

The general sequence for the histidine box motif is His-Xaa $_{(2-3)}$-His-His (SEQ ID NO: 29 and SEQ ID NO: 30).

Similar to other microsomal ω-6 desaturases, the deduced protein sequence of the ghFAD2-1 clone lacks a recognisable signal sequence for targeting to the endoplasmic reticulum (ER), and it is possible that the gene products may be translocated into the ER post-translationally, as in the case of the rat liver Δ9 stearoyl-CoA desaturase (Thiede *et al.*, 1985). A lysine-rich carboxyl-terminal motif, present in the *A. thaliana* FAD3 enzyme, which has been suggested to represent the retention signal of integral membrane proteins in the ER (Jackson *et al.*, 1990), is not present in the cotton microsomal ω-6 fatty acid desaturases.

Near the C-terminal of the ghFAD2-1 ORF there exists a stretch of six continuous glycine residues, which distinguish this amino acid sequence from other microsomal ω-6 desaturases (Figure 7).

Hydropathy plot analysis of the amino acid sequence encoded by ghFAD2-1 reveals six transmembrane helices. Interestingly, the three histidine boxes proposed to be iron-binding sites during catalysis, are not located in the region of transmembrane helices.

EXAMPLE 9

Expression of the ghFAD2-1 and ghFAD2-2 genes during cotton seed development

Northern hybridisations of a labelled ghFAD2-1 probe to cotton RNA were carried out under the same conditions as described hereinabove. As shown in Figure 8B, ghFAD2-1 expression was detected in the earliest embryos sampled (i.e. at 25 DAA). The detectable level of mRNA rapidly increased during embryo development, with the highest expression being at 30 DAA. Significantly lower amounts of transcripts were detected in the embryos nearer maturity, at 45 DAA or later. No transcripts of the ghFAD2-1 were detectable in cotton leaf tissues. The pattern of mRNA accumulation data presented herein suggest that the *ghFAD2-1* gene is expressed during the period of maximum storage lipid synthesis and accumulation in cotton, consistent with the increased linoleic acid accumulation during the same period. Accordingly, these data indicate that the ghFAD2-1 gene possibly plays a major role in the desaturation of cottonseed oil.

In contrast, very low expression of ghFAD2-2 is detected in both leaf and embryo tissues (Figure 8C). These data suggest that the ghFAD2-2 gene contributes in only a minor way to total fatty acid Δ12-desaturase activity in cotton seed, and, as a consequence, expression of this gene is minor contributing factor to the desaturation of cotton seed oil.

EXAMPLE 10

Organisation of the Oleoyl-PC Δ12 desaturase gene family

We analysed the organisation of the cotton oleoyl-PC Δ12 desaturase gene family, by Southern blot hybridisation of cotton genomic DNA samples, using labelled DNA probes consisting of the 3'-UTR of the ghFAD2-1 and ghFAD2-2 clones, or

alternatively, the entire coding regions of these cDNA clones, under high stringency hybridisation conditions, essentially as described hereinabove.

Genomic DNAs tested were from *G. barbadense*, *G. hirsutum* (cv Deltapine-16), *G. hirsutum* (cv Siokra), *G. herbaceum*, *G. raimondii*, and *G. robinsonii*. The cotton DNAs were digested with *Eco*RI, and *Hin*dIII.

Figure 9-A represents a Southern blot hybridised to a ghFAD2-1-specific fragment based on the sequence of the 3'-UTR of ghFAD2-1. In both *Eco*RI and *Hin*dIII digests, there are two distinct fragments in tetraploids (lane 1-3), one of which is present in each of the diploids (lane 4-6). Of the two fragments present in the tetraploid species, the smaller fragment has a similar size as the fragement of *G. herbaceum* (lane 4), whilst the larger fragment is similar to that of *G. raimondii* (lane 5). A single fragment is also present in *G. robinsonii*, the C-genome diploid species.

Figure 9-B represents a Southern blot using the more conserved coding region of ghFAD2-1 as a probe. Five *Eco*RI fragments, and four *Hin*dIII fragments are detectable in tetraploid cottons (lanes 1-3), whilst only two fragments are discernible in the diploids (lanes 4-6). However, one *Hin*dIII fragment (the third fragment from top in lanes 1-4; and the second fragment in lane 6) appears to be at least twice as strong as the other fragments, and presumably contains multiple copies of the gene. Therefore, we suggest that there are at least five members of the microsomal ω-6 desaturase gene family in each of the tetraploid cotton species. Furthermore, it appears that the A-genome diploid species, *G. herbaceum* (lane 4), has at least three genes, and the D-genome diploid species, *G. raimondii* (lane 5), has two genes, in this gene family.

The inconsistent fragment patterns obtained for *Eco*RI-digested and *Hin*dIII-digested *G. robinsonii* DNA (lane 6) may be a consequence of incomplete digestion of the *G. robinsonii* genomic DNA. In this respect, the pattern of *Hin*dIII

fragements observed for *G. robinsonii* are similar to those observed fro the A-genome diploid species.

In summary, Southern blot analyses indicate that there are at least five closely related microsomal ω-6 desaturase genes in each of two tetraploids, *G. barbadense* and *G. hirsutum*. The conservation of polymorphic restriction fragment patterns in tetraploid genomes and those representing their putative progenitors was sufficient to allow the assignment of each of the fragments in allotetraploid to their respective subgenomes. We estimate that, for tetraploid cotton, at least three genes (copies) were derived from the A-genome, and at least two genes may be derived from the D-genome. The *ghFAD2-1* gene appears to be a single-copy gene in diploid cotton species, indicating that a gene duplication probably did not occur in cotton.

The promoter sequence of the cotton *ghFAD2-1* gene is provided in SEQ ID NO: 7. In this respect, SEQ ID NO: 7 contains sufficient nucleotide sequence to confer expression on a structural gene to which it is operably connected in the cottonseed. In particular, the 5006 nucleotides of SEQ ID NO: 7 includes 3784 nucleotides upstream of the transcription start site of the *FAD2* gene (position 3785), the first intron of the gene (nucleotides 3889 to 4998) and the entire 5'-untranslated region (UTR) of the *FAD2* gene (nucleotides 3785 to 5006).

EXAMPLE 11

Gene constructs for silencing fatty acid biosynthesis genes

Gene constructs were produced to facilitate a reduction in the expression of the endogenous cotton fatty acid Δ9-desaturase gene (ghSAD-1), and the endogenous cotton fatty acid Δ12-desaturase gene (ghFAD2-1). The gene

constructs used the ghSAD-1 and ghFAD2-1 cDNA sequences, in the antisense orientation, or as inverted-repeat sequences having self-complementarity.

Construction of the pBI-Lectin binary plasmid

To confer seed-specific expression on gene fragments comprising antisense and inverted-repeat sequences of these genes, the soybean lectin promoter was used. A *Not*I adaptor sequence having a *Sma*I site was produced by annealing oligonucleotides having the nucleotide sequences, 5′-GGCCCGGG-3′ (SEQ ID NO: 13) and 5′GGCCCCCG-3′ (SEQ ID NO: 14) using standard conditions. This adaptor was ligated into the *Not*I site of the plasmid pGLe-10 (Cho, 1995), containing the soybean lectin promoter and terminator sequences. The plasmid was subsequently digested using *Eco*RI and *Hin*dIII, and a fragment comprising both the lectin promoter and lectin terminator sequences was isolated, and ligated into pBI121 that had been digested using *Eco*RI and *Hin*dIII, thereby replacing the CaMV 35S-GUS-NOS chimeric gene of pBI121 with the soybean lectin promoter/terminaotr-containing fragment. The components of this modified pBI-Lectin binary vector is illustrated in Figure10.

Gene fragments were cloned into the *Sma*I site of the pBI-Lectin binary vector, which site was present in the *Not*I adaptor. To facilitate such cloning steps, the pBI-Lectin binary vector was predigested with *Sma*I and dephosphorylated using calf intestinal phosphatase (CIP) enzyme (Pharmacia).

Fatty acidΔ9-desaturase antisense gene constructs

Antisense gene constructs targeted against the Δ9-desaturase gene comprise the full length ghSAD-1 cDNA clone, cloned in the antisense orientation as a blunt-ended *Not*I fragment, into the *Sma*I site of the pBI-Lectin binary vector.

Fatty acidΔ9-desaturase inverted repeat gene constructs

An inverted repeat of the 5′-terminal region of the cotton ghSAD-1 clone was produced by PCR amplification, according to the following description. A fragment

of approximately 500 bp in length derived from the of 5′-end of the cotton ghSAD-1 cDNA clone was amplified using the following primers:

(i) Primer 9s1 (SEQ ID NO: 15):5′-TTTTAATGCCATCGCCTCG-3′; and

(ii) Primer 9a1 (SEQ ID NO: 16): 5′-CTTCAGCAGTCCAAGCCCTG-3′.

The amplified gene fragment was cloned into Teasy® vector (Promega). One clone having the amplified gene fragment in a desired orientation was selected, and the gene fragment was released by digestion of the plasmid DNA using *Sal*I and *Apa*I. Concurrently, the full-length ghSAD-1 cDNA clone having the desired orientation was amplified by PCR, using the reverse primer present in the pBluescript vector, and the 9a1 primer *supra*, and this clone was linearised by digestion withthe enzymes *Sal*I and *Apa*I. The 500 bp *Sal*I/*Apa*I fragment, containing only the 5′-end of the cotton ghSAD-1 cDNA clone, was ligated to the linearised full-length cDNA in pBluescript. This ligation produced a partial inverted repeat sequence comprising the full-length ghSAD-1 clone extended at its 3′-end by 500 bp of the 5′-end nucleotide sequence in the reverse orientation. Accordingly, the ligation product contained a perfect inverted repeat of the 500 bp 5′-end nucleotide sequence. To release this inverted repeat sequence, the ligation product was digested using the restriction enzymes *Apa*I and *Xba*I. The inverted repeat sequence was then end-filled using Klenow fragment, and the blunt-ended fragment was ligated into the *Sma*I site of the pBI-Lectin binary vector, between the lectin promoter and lectin terminator sequences.

Fatty acidΔ12-desaturase antisense gene constructs

The full-length cDNA ghFAD2-1 clone was amplified by PCR, using a pair of amplification primers, as follows:

Primer 12s1 (SEQ ID NO: 17):5′-CCTGGCGTTAAACTGCTTTC-3′; and

Primer 12a2 (SEQ ID NO: 18):5′-CCATATAGTTTATTAATATAACAC-3′.

The amplification product was cloned into either the Teasy® vector, or alternatively, the T® vector (Promega). The full-length ghFAD2-1 insert was released from the Teasy® vector by digestion with *Not*I, end-filled using Klenow fragment, and ligated into the *Sma*I site of the pBI-Lectin binary vector, between the lectin promoter and lectin terminator sequences. A clone comprising the ghFAD2-1 cDNA clone in the antisense orientation was selected by nucleotide sequence determination.

Fatty acid Δ12-desaturase inverted repeat gene constructs

An inverted repeat of the 5′-terminal region of the cotton ghFAD2-1 clone was produced by PCR amplification, according to the following description.

The full-length ghFAD2-1 cDNA in T® vector with the desired orientation was selected using the forward primer on the T vector, and primer 12a1 (SEQ ID NO: 19; 5′-TATGTTGCAAGTAGGTGATC-3′). Then it was linearized by digestion of the plasmid DNA using *Not*I (the T® vector contains only one *Not*I site in the polylinker region).

Concurrently, a fragment of approximately 850 bp in length derived from the of 5′-end of the cotton full-length ghFAD2-1 cDNA clone was amplified by PCR, using the primers 12s1 and 12a1, and the amplified DNA was cloned into the Teasy® vector. The amplified fragment was digested using the enzyme *Not*I.

The 850 bp *Not*I fragment, containing only the 5′-end of the cotton ghFAD2-1 cDNA, was ligated to the *Not*I site in the T® vector containing the full-length ghFAD2-1 cDNA clone. This ligation produced a partial inverted repeat sequence comprising the full-length ghFAD2-1 clone extended at its 3′-end by about 850 bp of the 5′-end nucleotide sequence, in the reverse orientation. Accordingly, the ligation product contained a perfect inverted repeat of about 850 bp of 5′-end nucleotide sequence. To release this inverted repeat sequence, the ligation product was digested using the restriction enzymes *Apa*I and *Sac*I. The inverted repeat sequence fragment was then end-filled using Klenow fragment, and the

blunt-ended fragment was ligated into the *SmaI* site of the pBI-Lectin binary vector, between the lectin promoter and lectin terminator sequences.

An additional Δ12-desaturase gene silencing construct was produced that comprised an inverted-repeat produced from about 92 bp of the 5'-UTR region of the genomic *ghFAD2-1* gene (SEQ ID NO: 7). In this gene construct, the 5'-UTR of the *ghFAD2-1* gene was separated by nucleotide sequence from the first intron of the *ghFAD2-1* gene. The inverted repeat sequence was placed operably in connection with regulatory sequences from the *ghFAD2-1* gene (i.e. sequences derived from SEQ ID NO: 7). This gene construct is referred to hereinafter as an "intron-interrupted UTR inverted-repeat gene construct".

To produce the intron-interrupted UTR inverted-repeat gene construct, nucleic acid was amplified by PCR from *G. hirsutum* cv Coker 315 genomic DNA, using the following amplification primers:

(i) PITSal (SEQ ID NO: 20):
5'-ACGCGTCG**ACGTGTGTTACAAAATGGACCGAA**-3'; and

(ii) PITBam (SEQ ID NO: 21):
5'-CGCGGATCC**GCTGGCTGGACACGCAAGAAGCA**-3'.

Nucleotide residues of the primer PITSal *supra* shown in bold typeface correspond to nucleotides 2569 to 2592 of SEQ ID NO: 7. This primer has a 5'- localized *SalI* site. Nucleotide residues of the primer PITBam *supra* shown in bold typeface are complementary to nucleotides 4981 to 5003 of SEQ ID NO: 7. This primer has a 5'- localized *Bam*HI site, which is incorporated into the 3'-end of the amplified DNA. Accordingly, DNA amplified using this primer pair comprises a 5'-*SalI* site and a 3'-*Bam*HI site to facilitate sub-cloning.

The amplified gene fragment included approximately 1.2kb of nucleotide sequence upstream of the transcription start site of the *ghFAD2-1* gene. This is sufficient sequence to function as a promoter in cottonseed. The amplified DNA fragment also includes the entire 5'-UTR and first intron of the *ghFAD2-1* gene.

The amplified DNA fragment was digested with *Sal*I and *Bam*HI, and cloned directionally into the corresponding sites of the vector pBI101.2, to yield an intermediate *ghFAD2-1* promoter-intron construct.

To produce the inverted repeat within the 5'-UTR, about 92 bp of 5' UTR sequence from the *gh*FAD2-1 gene, without intron sequence, was amplified from cottonseed cDNA, using the following PCR primers:-

(i) Usac (SEQ ID NO: 22):
(5'-CGA**GCTCCCCCTCCGCTCCATACCACT**-3'); and

(ii) Ubam (SEQ ID NO: 23)
(5'-CGCGGATCCGCTGG**CTTTAAAGAAAGCAGTT**-3').

Nucleotide residues of the primer Usac *supra* shown in bold typeface correspond to nucleotides 3796 to 3820 of SEQ ID NO: 7. This primer has a 5'- localized *Sac*I site. Nucleotide residues of the primer Ubam *supra* shown in bold typeface are complementary to nucleotides 3872 to 3888 of SEQ ID NO: 7. This primer has a 5'- localized *Bam*HI site, which is incorporated into the 3'-end of the amplified DNA. Accordingly, DNA amplified using this primer pair comprises a 5'-*Sac*I site and a 3'-*Bam*HI site to facilitate sub-cloning into the intermediate *ghFAD2-1* promoter-intron construct.

The amplified DNA fragment was cloned, in the inverted orientation relative to its orientation in the *ghFAD2-1* gene, downstream of the intron in the intermediate *ghFAD2-1* promoter-intron construct. Accordingly, the final intron-interrupted UTR inverted-repeat gene construct thus comprised the following features:

(i) nucleotide residues 2569-3785 of SEQ ID NO: 7 comprising sequences sufficient to confer expression in cottonseed and the transcription start site of the *ghFAD2-1* gene;

(ii) a part of the 5'-UTR of the *ghFAD2-1* gene corresponding to nucleotides 3785-3888 of SEQ ID NO: 7;

(iii) the first intron of the *ghFAD2-1* gene corresponding to nucleotides 3889-4998 of SEQ ID NO: 7 placed downstream of (ii);

(iv) a part of the 5'-UTR of the *ghFAD2-1* gene corresponding to nucleotides 4999-5003 of SEQ ID NO: 7 placed downstream of (iii); and

(v) a part of the 5'-UTR of the *ghFAD2-1* gene corresponding to nucleotides 3796-3888 of SEQ ID NO: 7 placed downstream of (iv) and in the inverted orientation relative to (ii).

EXAMPLE 12

Transformation of cotton plants

Transgenic cotton plants (*G. hirsutum* cv. Coker 315) were generated by *Agrobacterium-mediated* infection, and selection on medium containing kanamycin sulphate, by a modification of the method described by Cousins *et al.* (1991).

Briefly, cotton seedlings were germinated aseptically on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), solidified using phytagel (Sigma). Seedlings were maintained under low light conditions at 28°C. Callus was initiated from cotyledon explants of 10-14 day old seedlings, on callus initiation medium [MS macro and micro elements; B5 vitamins (Gamborg, 1968); 100 mg/l myo-inositol; 30g/l glucose; 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D); 0.1 mg/l kinetin; and 0.93 g/l magnesium chloride], solidified using 2 g/l phytagel.

Explants derived from about 20 seedlings was infected with *A. tumefaciens* strain AGL1 which had been transformed with the relevant gene construct (*supra*) by standard electroporation. Following 2 days of cocultivation in the presence of *A. tumefaciens*, the cotton explants were transferred to MS medium containing 0.1 mg/l 2,4-D, 0.1 mg/l kinetin, 50 mg/l kanamycin sulphate, and 250 mg./l cefotaxime, and incubated at 28°C for six weeks.

Healthy calli were then transferred to MS medium containing 5 mg/l 6-(γ,γ-dimethylallylamino)-purine (2ip), 0.1 mg/l naphthalene acetic acid (NAA), 25 mg/l kanamycin, and 250 mg/l cefotaxime for a second selection period of six weeks at 28°C.

Developing embryos were maintained and germinated into plantlets as described by Cousins *et al.* (1991). Briefly, following the second selection period, the central soft parts of the calli were transferred onto solidified MS medium, without addded phytohormone or antibiotic, to initiate embryogenesis. Embryogenic calli formed after about six to ten weeks of incubation at 28°C. The embryogenic calli were transferred to embryo development medium comprising MS medium containing an additional 1.9 g/l potassium nitrate. Embryos continue to develop on this medium, and can be removed, and germinated on SH medium (Stewart, 1977) solidified with phytagel, to produce transgenic cotton plantlets.

Transgenic cotton plantlets (T1 generation) were transferred to soil, and maintained in a glasshouse, once leaves and roots developed.

EXAMPLE 13

Characterisation of transgenic cotton plants

No obvious phenotypic differences were observed between the transgenic lines and isogenic non-transformed control plants. Genomic DNA was prepared from young leaves of regenerated cotton (T1) plants, essentially as described herein above.

The presence of the respective gene silencing transgene in each cotton plant was determined by PCR, using genomic DNA as a template, and the following primers:

Primer 3Lec-s1 (SEQ ID NO: 24): 5′-CATGTGACAGATCGAAGGAA-3′;

and

Primer3Lec-a1 (SEQ ID NO: 25): 5′-ATCTAATTATTCTATTCAGAC-3′.

This process amplifies an approximately 300 bp DNA fragment comprising the transcriptional terminator of the soybean lectin gene. Accordingly, amplification only occurs from plant DNA containing the introduced chimeric genes. Further confirmation that the amplified fragment was indeed the soybean lectin terminator was obtained by performing Southern blot hybridization analysis, using the isolated soybean lectin terminator as a probe, as described herein above.

To confirm the presence of the fatty acid biosynthesis antisense and/or inverted repeat sequences in the transgenic cotton plants, genomic DNA derived from each putative transgenic plant was digested with *Hin*dIII, separated on a 0.7% (w/v) agarose gel, blotted onto a Hybond-N+ nylon membrane, and probed with an [α-^{32}P]dCTP-labelled gene-specific DNA fragment. The conditions of hybridization and the following washing was the same as described hereinabove. Data presented in Table 6 indicate the copy number of transgenes in some representative lines that were analyzed.

TABLE 6

Copy number of transgenes in T1 cotton plants

Transgene copy number	Plant line		Plant line	
	Δ9-desaturase inverted repeat construct	Δ9-desaturase antisense construct	Δ12-desaturase inverted repeat construct	Δ12-desaturase antisense construct
1	95-6 95-37 95-40 95-43 95-49 95-55 95-70 95-72 95-75 95-79 95-87 95-146	9A-17 9A-38 9A-148 9A-163 9A-183	125-21 125-23 125-26 125-27 125-31 125-33 125-50 125-60 125-62 125-82 125-92 125-103 125-117 125-128	12A-4 12A-11 12A-43 12A-45 12A-48 12A-59 12A-73 12A-74 12A-130
2	95-31 95-91 95-112 95-120 95-136	9A-89 9A-127	125-2 125-54 125-58 125-81 125-96	12A-41 12A-111
3	95-21 95-22	9A-192	125-12 125-32	12A-60 12A-82

	95-51 95-61 95-80 95-96		125-79 125-114 125-125	12A-124
4	95-65	9A-118	125-10	12A-117
5	95-23 95-57 95-150	9A-119 9A-189	125-83	
6		9A-57	125-87	
7				12A-68
8			125-121	
9			125-120	
10				12A-34
12				12A-56

EXAMPLE 14

Characterisation of oils in transgenic T2 seed containing inverted repeats of a fatty acid Δ9-desaturase (stearoyl-ACP Δ9-desaturase) gene

The seeds produced by independent transgenic (T1) plants containing inverted repeats of the cotton fatty acid Δ9-desaturase gene, and a non-transformed isogenic *G. hirsutum* Coker cotton, were analyzed for their fatty acid composition. Samples consisting of three pooled T2 seeds derived from a number of individual T1 plants. Fatty acid methyl esters were prepared as described by Bligh and Dyer (1959). Methyl esters were separated by gas chromatography (GC), using a Hewlett-Packard 5890 gas chromatograph fitted with a fused silica capillary column (HP-FFAP, 0.53 mm x 30 m). Fatty acids were identified by reference to chemical standards as described herein above.

Data are presented in Table 7. Because T1 plants were generally hemizygous for the introduced transgene, the T2 seeds were segregating for that transgene. Accordingly, the data for pooled T2 seed samples shown in Table 7 are an averaging of fatty acid levels for seeds that are either homozygous or hemizygous for the transgene, or lack the transgene altogether.

Increased stearic acid

Expression of the inverted repeat of the 5'-end of ghSAD-1 in cotton appeared to decrease the level of the stearoyl-ACP Δ9-desaturase enzyme activity in cotton, as suggested by the elevated level of stearic acid in transgenic T2 seed. As summaried in Table 7, at least half of the total 22 individual T1 transgenic plants produced seed having elevated levels of stearic acid when compared to isogenic wild-type seed. Stearic acid was increased up to about 38% of total seed lipid (line 95-62), which represents an approximately 15-fold increase in the level of this fatty acid.

Decreased oleic acid

Without being bound by any theory or mode of action, the increased production of stearic acid in transgenic lines, which results from the decreased expression of the endogenous fatty acid Δ9-desaturase (stearoyl-ACP Δ9-desaturase) gene, is at the expense of oleic acid and linoleic acid production, because these fatty acids are all products of fatty acid Δ9-desaturase (stearoyl-ACP Δ9-desaturase) enzyme activity. In this respect, oleic acid was reduced, from 15% of total seed lipid in the non-transgenic cotton, to only 5.1% in the line 95-62, representing a decrease in oleic acid content of up to about 65% in the transgenic lines.

Decreased linoleic acid

Linoleic acid was also reduced, from 57.4% of total seed lipid in the non-transgenic, to 37.8% in line 95-62, representing a decrease in linoleic acid content of up to about 33% in the transgenic lines.

Decreased palmitic acid

Unexpectedly, and favourably, palmitic acid was significantly reduced in the T2 seed of transgenic cotton containing the inverted repeat of the 5'-end of ghSAD-1, particularly in those lines having increased stearic acid. In particular, the level of palmitic acid in the seed of trasngenic plants was reduced to only 14.7% of total seed lipid in line 95-62, compared to 24.4% of total seed lipid for isogenic non-transformed plants. This represents a decrease in palmitic acid content of up to about 45% in the transgenic lines.

TABLE 7

Fatty acid contents of T1 transgenic cotton comprising an inverted repeat of the 5'-end of the ghSAD-1 clone

Plant line	Fatty acid composition (%)					SDP
	Palmitic	Stearic	Oleic	Linoleic	Linolenic	
Coker control	24.4	2.7	15.0	57.4	0.2	0.97
95-70	26.1	2.4	14.5	56.6	0.2	0.97
95-33	25.4	2.5	15.4	56.3	0.2	0.97
95-128	22.5	2.6	14.2	60.3	0.2	0.97
95-146	25.1	2.5	15.2	56.8	0.2	0.97
95-112	26.4	2.5	15.2	55.5	0.2	0.97
95-78	24.6	2.6	14.7	57.6	0.2	0.97
98-92	24.3	2.6	16.3	56.4	0.2	0.97
95-43	25.6	2.7	16.5	54.7	0.2	0.96
95-13	22.9	2.7	12.9	61.2	0.1	0.96
95-95	24.3	2.9	15.6	56.8	0.2	0.96
95-65	25.4	2.9	14.7	56.5	0.2	0.96
95-120	24.9	3.0	15.8	55.8	0.2	0.96
95-90	20.6	3.4	13.7	61.8	0.2	0.96
95-21	23.1	4.0	13.4	58.9	0.2	0.95
95-61	24.1	4.2	13.5	57.8	0.2	0.94
95-72	26.0	4.7	14.0	54.6	0.2	0.94

95-51	24.5	5.0	13.6	56.3	0.2	0.93
95-136	24.3	6.6	13.3	55.2	0.2	0.91
95-40	22.7	8.8	12.0	55.8	0.1	0.88
95-98	19.6	10.9	11.6	57.1	0.2	0.86
95-78	22.2	13.0	10.6	53.3	0.2	0.83
95-99	20.5	14.1	9.6	55.0	0.1	0.82
95-6	23.6	14.1	10.1	51.1	0.2	0.81
95-125	21.3	15.2	11.1	51.3	0.2	0.80
95-55	20.1	20.2	8.0	51.6	0.0	0.75
95-37	18.8	23.4	7.9	48.4	0.4	0.71
95-150	19.0	28.0	5.8	45.4	0.3	0.65
95-62	14.7	38.2	5.1	37.8	1.6	0.54

EXAMPLE 15

Characterisation of oils in transgenic seed that is homozygous for the transgene containing an inverted repeat of a fatty acid Δ9-desaturase gene

T2 Lines that are homozygous for the introduced inverted repeat of the cotton fatty acid Δ9-desaturase gene were identified by selection. The fatty acid compositions of individual T2 seeds (15 in total), derived from several representative T1 plants that differed widely in their stearic acid content, were analyzed as described in the preceding example. Data are presented in Table 8.

It is clear that different transgenic events are associated with different levels of reduction in Δ9-desaturase activity (as indicated by SDP) and corresponding different levels of accumulation of the stearic acid substrate. This variation is explainable by different transgene copy numbers and different genomic integration locations of the transgenes. By producing a sufficient number of independent transgenics, any particular desired stearic acid content between the normal upper limit for cotton (3%) and approximately 35% can therefore be obtained (see Figure 11).

To confirm that the observed Δ9-desaturase gene silencing was heritable, additional T2 seed, and T3 seed, were obtained from plants exhibiting the most severe silencing phenotype, in particular, the independent T1 plants having the highest levels of stearic acid in their seed and designated 95-62 and 95-150 (Table 7). The fatty acid compositions of 15 individual T2 seed and 12 individual T3 seed from these lines were determined as described in the previous example. The T2 segregation patterns were consistent with a single transgene insertion. Data presented in Table 9 demonstrate that the high level silencing observed in the T2 seeds as shown in Table 8 is inherited in the T3 generation, as evidenced by the heritability of the high stearic acid and low oleic acid and low linoleic acid and low palmitic acid phenotypes.

TABLE 8

Fatty acid composition (% of total fatty acids) of 15 individual T2 seeds from four T1 plants of cotton transformed with the Δ9-desaturase inverted-repeat gene silencing construct and from untransformed Coker control plants.

T1 plant number	T2 seed number	Fatty acid composition (%)					SDP
		Palmitic	Stearic	Oleic	Linoleic	Linolenic	
Coker control	1	25.1	2.6	15.2	56.6	0.2	0.97
	2	25.1	2.6	14.9	57.0	0.2	0.97
	3	24.3	2.2	14.7	58.3	0.2	0.97
	4	25.2	2.5	14.4	57.4	0.2	0.97
	5	24.9	2.3	15.0	57.4	0.2	0.97
	6	25.2	2.6	14.8	56.9	0.2	0.97
	7	25.4	2.8	15.7	55.6	0.2	0.96
	8	23.9	2.7	15.1	58.0	0.2	0.96
	9	24.0	2.9	16.2	56.4	0.2	0.96
	10	24.4	2.8	15.0	57.3	0.2	0.96
	11	23.3	3.0	14.7	58.5	0.2	0.96
	12	23.1	3.2	14.9	58.2	0.2	0.96
	13	23.9	2.9	14.9	57.8	0.2	0.96
	14	22.9	2.9	14.4	59.3	0.2	0.96
	15	25.0	2.7	15.5	56.3	0.2	0.96
	Mean	24.4	2.7	15.0	57.4	0.2	0.96
95-6	1	25.9	2.5	15.0	56.1	0.2	0.97
	2	25.4	3.1	13.4	57.6	0.2	0.96
	3	24.7	3.3	14.7	56.7	0.2	0.96
	4	28.5	2.6	14.3	54.2	0.2	0.96
	5	25.9	3.1	13.0	57.5	0.2	0.96
	6	24.5	8.4	11.9	54.5	0.2	0.89
	7	25.8	8.5	12.3	52.7	0.2	0.88
	8	25.4	9.3	11.4	53.1	0.2	0.87

	9	23.6	11.6	11.8	52.1	0.2	0.85
	10	23.3	12.1	11.2	52.6	0.2	0.84
	11	23.6	12.1	10.6	52.8	0.2	0.84
	12	24.8	12.9	10.0	51.4	0.2	0.83
	13	23.6	12.5	11.2	51.8	0.2	0.83
	14	22.7	14.5	10.4	51.4	0.2	0.81
	15	23.6	16.0	9.9	49.4	0.2	0.79
	Mean	24.8	8.8	12.1	53.6	0.2	0.88
95-37	1	23.9	2.6	13.9	59.0	0.3	0.97
	2	23.9	3.0	14.7	58.0	0.2	0.96
	3	23.7	2.7	14.7	58.5	0.2	0.96
	4	23.4	2.9	16.0	57.4	0.2	0.96
	5	23.9	2.8	14.6	58.2	0.2	0.96
	6	19.0	21.6	8.0	50.0	0.3	0.73
	7	19.4	21.1	8.5	49.7	0.3	0.73
	8	19.2	22.4	8.8	48.3	0.2	0.72
	9	18.4	22.2	10.0	47.9	0.3	0.72
	10	18.1	23.7	7.5	49.2	0.3	0.71
	11	18.2	24.8	7.4	48.1	0.3	0.69
	12	18.0	24.9	7.6	47.9	0.3	0.69
	13	17.5	25.1	8.1	47.7	0.3	0.69
	14	18.6	25.1	7.3	47.3	0.4	0.69
	15	17.9	27.8	7.2	45.5	0.3	0.66
	Mean	20.2	16.8	10.3	51.5	0.3	0.79
95-62	1	24.2	3.4	32.7	39.1	0.2	0.95
	2	24.0	11.1	19.8	43.8	0.3	0.85
	3	22.6	12.8	16.6	47.0	0.2	0.83
	4	21.6	14.4	11.4	51.5	0.3	0.81
	5	22.4	14.2	16.8	45.5	0.2	0.81

	6	20.3	16.8	12.5	49.3	0.3	0.79
	7	18.4	20.5	12.5	47.4	0.2	0.75
	8	20.2	19.8	8.8	49.9	0.3	0.75
	9	19.6	20.6	10.9	47.5	0.3	0.74
	10	18.3	23.7	9.1	47.4	0.3	0.71
	11	19.0	24.0	8.4	46.9	0.3	0.70
	12	18.6	24.4	8.4	47.1	0.2	0.70
	13	18.2	26.3	9.4	44.3	0.3	0.67
	14	17.0	30.8	6.5	43.8	0.3	0.62
	15	14.7	34.3	7.3	41.4	0.4	0.59
	Mean	19.9	19.8	12.7	46.1	0.3	0.75
95-150	1	26.2	2.3	16.3	54.8	0.1	0.97
	2	25.0	3.0	13.3	58.2	0.1	0.96
	3	25.0	3.0	14.0	57.5	0.2	0.96
	4	23.5	10.5	14.1	51.1	0.2	0.86
	5	22.4	14.6	11.8	50.2	0.2	0.81
	6	22.5	14.7	10.3	51.5	0.2	0.81
	7	22.2	15.8	9.8	51.3	0.2	0.80
	8	21.6	16.0	10.2	51.2	0.2	0.79
	9	22.6	16.6	9.9	49.7	0.2	0.78
	10	20.5	20.7	8.2	49.3	0.2	0.74
	11	21.0	20.6	8.2	48.8	0.2	0.74
	12	20.8	21.2	7.0	49.8	0.2	0.73
	13	20.8	20.9	7.9	49.1	0.2	0.73
	14	19.9	23.8	6.8	48.1	0.2	0.70
	15	19.5	24.8	7.7	46.5	0.2	0.69
	Mean	22.2	15.2	10.4	51.1	0.2	0.80

TABLE 9

Fatty acid composition (% of total fatty acids) of 12 individual T3 seeds from two independently derived T2 plants of cotton transformed with the Δ9-desaturase inverted-repeat gene silencing construct.

T1 line	T3 seed No.	Fatty acid composition (%)					SDP
		Palmitic	Stearic	Oleic	Linoleic	Linolenic	
95-62	1	16.6	32.9	7.3	41.2	0.3	0.60
	2	13.5	37.0	6.6	37.9	2.4	0.56
	3	17.6	32.2	7.3	41.1	0.1	0.60
	4	15.2	36.1	9.5	36.6	0.5	0.56
	5	15.8	37.3	7.9	36.4	0.4	0.54
	6	16.1	35.3	6.4	39.9	0.4	0.57
	7	16.8	29.2	8.5	42.3	1.3	0.64
	8	15.7	35.8	6.6	39.5	0.4	0.57
	9	15.8	35.6	7.9	38.3	0.4	0.57
	10	16.6	33.4	8.6	39.1	0.4	0.59
	11	16.5	33.6	7.5	40.1	0.4	0.59
	12	15.7	36.4	6.8	38.6	0.4	0.56
	Mean	*16.0*	*34.6*	*7.6*	*39.3*	*0.6*	*0.58*
95-150	1	17.7	26.5	7.5	46.7	0.2	0.67
	2	18.5	24.6	7.4	47.8	0.3	0.69
	3	16.1	31.3	6.5	44.2	0.2	0.62
	4	20.7	17.8	9.1	51.0	0.2	0.77
	5	16.3	31.1	6.6	44.4	0.2	0.62
	6	18.2	27.1	7.1	46.7	0.1	0.67
	7	19.3	21.9	7.4	49.6	0.7	0.73
	8	17.6	27.7	7.8	45.9	0.2	0.66
	9	15.8	32.0	7.1	43.1	0.2	0.61

10	17.6	29.0	6.8	44.9	0.2	0.64
11	15.7	33.8	5.7	42.9	0.2	0.59
12	18.0	25.4	7.2	47.8	0.2	0.69
Mean	*17.6*	*27.3*	*7.2*	*46.3*	*0.2*	*0.66*

EXAMPLE 16

Fatty acid contents of oils from seed of transgenic T1 cotton plants carrying the *ghFAD2-1* inverted repeat gene construct

The seeds produced by independent transgenic (T1) plants containing an inverted repeat of the cotton fatty acid Δ12-desaturase gene, and a non-transformed isogenic *G. hirsutum* coker cotton, were analysed for their fatty acid composition. Samples consisting of three pooled T2 seeds derived from a number of individual T1 plants. Fatty acid methyl esters were prepared as described by Bligh and Dyer (1959). Methyl esters were separated by gas chromatography (GC), using a Hewlett-Packard 5890 gas chromatograph fitted with a fused silica capillary column (HP-FFAP, 0.53 mm x 30 m). Fatty acids were identified by reference to chemical standards as described hereinabove.

Data are presented in Table 10. Because T1 plants were generally hemizygous for the introduced transgene, the T2 seeds were segregating for that transgene. Accordingly, the data for pooled T2 seed samples shown in Table 10 are an averaging of fatty acid levels for seeds that are either homozygous or hemizygous for the transgene, or lack the transgene altogether.

Increased oleic acid

Expression of the inverted repeat of the 5'-end of ghFAD2-1 in cotton appeared to decrease the level of the oleoyl-PC Δ12 desaturase enzyme activity in cotton, as suggested by the elevated level of oleic acid in transgenic T2 seed. As summaried in Table 10, several independent transgenic lines exhibited increased oleic acid

content compared to non-transformed plants . The oleic acid content increased from 15% for non-transformed Coker cotton, to 77% for the transgenic line 125-23, representing an increase of up to about 5-fold to 5.5-fold for this fatty acid in cotton.

Decreased linoleic acid

Linoleic acid was reduced in transgenic cotton lines, from 57.4% of total seed lipid in the non-transgenic Coker cotton, to only 3.5% in line 125-50, representing a decrease in linoleic acid content of up to about 95% in the transgenic lines.

Decreased palmitic acid

Unexpectedly, and favourably, palmitic acid was significantly reduced in the T2 seed of transgenic cotton containing the inverted repeat of the 5'-end of ghFAD2-1. The level of palmitic acid in the seed of transgenic plants was reduced to only 16.8% of total seed lipid in line 125-23, compared to 24.4% of total seed lipid for isogenic non-transformed plants. This represents a decrease in palmitic acid content of up to about 35% to 37% in the transgenic lines.

TABLE 10

Fatty acid contents of ghFAD2-1-downregulated transgenic cotton

Plant line	Fatty acid composition (%)					ODP
	Palmitic	Stearic	Oleic	Linoleic	Linolenic	
Coker	24.4	2.7	15.0	57.4	0.2	0.79
125-26	23.8	2.6	13.2	60.0	0.1	0.82
125-54	24.5	2.5	13.2	59.6	0.0	0.82
125-8	24.0	2.6	13.7	59.3	0.2	0.81
125-83	25.0	2.7	13.7	58.2	0.2	0.81
125-60	25.3	2.4	13.8	58.0	0.2	0.81
125-51	23.4	2.5	14.0	59.7	0.2	0.81
125-127	24.0	2.7	14.1	58.8	0.2	0.81
125-82	24.9	2.2	14.6	58.1	0.0	0.80
125-125	25.6	2.6	14.2	57.5	0.2	0.80
125-10	23.2	2.9	14.3	59.2	0.2	0.81
125-13	24.4	2.6	14.6	58.0	0.2	0.80
125-129	24.1	2.4	15.2	57.9	0.2	0.79
125-84	23.9	2.9	15.3	57.5	0.2	0.79
125-117	23.8	2.4	15.5	57.8	0.2	0.79
125-12	23.9	2.8	16.4	56.4	0.2	0.78
125-131	23.9	1.9	24.3	49.6	0.1	0.67
125-79	22.2	2.7	32.2	42.4	0.2	0.57
125-120	23.5	2.4	32.6	40.9	0.2	0.56

125-130	20.7	2.7	47.3	28.8	0.2	0.39
125-33	18.7	3.1	51.2	26.4	0.2	0.34
125-121	21.7	2.2	58.5	17.1	0.2	0.23
125-62	17.6	2.3	66.0	13.5	0.3	0.17
125-7	16.0	2.6	73.7	7.2	0.2	0.09
125-81	20.9	2.6	68.9	7.0	0.2	0.09
125-85	20.8	2.7	71.5	4.4	0.2	0.06
125-124	18.7	1.8	72.5	6.4	0.2	0.08
125-128	18.5	2.4	74.3	4.3	0.1	0.06
125-1	18.1	2.7	73.7	44.9	0.2	0.06
125-96	19.4	2.2	74.1	3.8	0.2	0.05
125-50	18.9	2.3	74.7	3.5	0.2	0.05
125-114	17.8	2.2	75.1	4.4	0.1	0.06
125-23	16.8	1.4	77.0	4.4	0.2	0.06

It is well established for oilseed plant species that the fatty acid composition of a somatic embryoid reflects the fatty acid composition of the sexual embryo. For example, in soybean, the fatty acid composition of middle maturity somatic embryoids is predictive of the final oil composition of seed derived from plants that have been regenerated from these embryoids. Proceeding on this basis, somatic embryoids generated from transgenic soybean calli have previously been used as an indicator of the fatty acid composition of the seed derived therefrom (Cahoon *et al.*, 1999; Cahoon *et al*, 2000). This approach facilitates the early detection and rapid selection of transgenic lines having desired oilseed composition.

To demonstrate the effectiveness of the intron-interrupted UTR inverted-repeat gene construct (Example 11) in down-regulating expression of the Δ12-desaturase

gene, the fatty acid compositions of somatic embryoids regenerating from 18 independent primary transformed calli of Coker cotton were determined. Data presented in Table 11 support the conclusion that the *ghFAD2-1* gene encoding fatty acid Δ12-desaturase has been silenced in lines expressing the intron-interrupted UTR inverted-repeat gene construct.

In particular, the level of oleic acid has been enhanced in somatic embryoids carrying the intron-interrupted UTR inverted-repeat gene construct, compared to the levels typical for somatic embryoids derived from untransformed plants. Oleic acid constitutes as much as 66% of the total fatty acids in transformed embryoids (Table 11), compared to only about 15% for typical somatic embryoids derived from untransformed Coker cotton plants (Tables 7 and 8). On average, this represents about 3-fold enhanced oleic acid content.

Additionally, the level of linoleic acid is reduced in somatic embryoids, carrying the intron-interrupted UTR inverted-repeat gene construct, compared to the levels typical for somatic embryoids derived from untransformed plants. Linoleic acid constitutes only about 10-31% (average 23%) of the total fatty acids in transformed somatic embryoids (Table 11), compared to about 57.4% for typical somatic embryoids derived from transformed Coker cotton plants (Tables 7 and 8). On average, this represents more than 50% reduction in linoleic acid content.

Additionally, the level of palmitic acid is reduced in the transgenic somatic embryoids carrying the intron-interrupted UTR inverted-repeat gene construct, compared to somatic embryoids derived from untransformed plants. Palmitic acid constitutes on average about 20% of the total fatty acids in transformed embryoids (Table 11), compared to about 24.4% for somatic embryoids derived from untransformed Coker cotton plants (Tables 7 and 8). On average, this represents about 25% reduction in linoleic acid content.

Since similar results were obtained using different inverted repeat gene constructs, the modulation of fatty acid content obtained using inverted repeat sequences is

not dependent on the precise nature of the inverted repeat in the gene construct. However, the frequency of gene silencing that we detected was enhanced in transformants carrying the intron-interrupted UTR inverted-repeat gene construct compared to transformants carrying the coding region inverted-repeat, or transformants carrying antisense constructs.

TABLE 11

Fatty acid contents of embryoids carrying the *ghFAD2-1* intron-interrupted UTR inverted-repeat construct

Embryoid	Fatty acid composition (%)					ODP
No.	Palmitic	Stearic	Oleic	Linoleic	Linolenic	
1	19.9	6.4	45.1	23.4	3.8	0.38
2	19.3	4.5	46.5	27.9	1.4	0.39
3	19.5	3.3	63.6	10.8	2.2	0.17
4	22.1	5.5	48.9	20.1	3.4	0.32
5	20.8	5.8	56.0	15.0	1.7	0.23
6	20.3	7.7	49.7	18.2	2.8	0.30
7	22.0	4.4	45.0	24.4	4.2	0.39
8	15.7	3.2	26.3	28.6	26.3	0.68
9	23.1	6.1	42.9	22.9	3.9	0.38
10	18.5	2.1	55.2	24.0	0.1	0.30
11	21.6	5.1	45.5	25.5	1.5	0.37
12	23.2	5.4	41.2	27.6	1.8	0.42
13	19.9	2.3	49.6	28.0	0.0	0.36
14	19.0	2.1	47.5	31.2	0.1	0.40
15	19.5	2.3	65.6	10.8	1.7	0.16
16	18.1	3.6	57.7	20.7	1.9	0.28
17	22.0	4.4	45.0	26.4	2.4	0.39
18	21.4	4.6	43.1	28.6	1.7	0.41
Mean	20.3	4.4	48.6	23.0	3.4	0.35

A proportion of the somatic embryoids carrying the intron-interrupted UTR inverted-repeat construct were allowed to regenerate into T1 plants. The fatty acid compositions of T2 seed from those regenerated plants were determined. Data presented in Table 12 show the fatty acid composition of 15 individual T2 seeds. Those data indicate that, as with the somatic embryoids from which the plants were derived, the level of oleic acid is enhanced, and the levels of palmitic acid and linoleic acid are reduced, in the T2 seeds of embryoid-derived T1 plants.

These data demonstrate that distinct gene silencing constructs comprising inverted repeats of fatty acid desaturase genes are effective in predictably modulating the fatty acid composition of cottonseed. The expression of such gene constructs may be placed operably under the control of distinct seed-operable promoter sequences.

TABLE 12

Fatty acid contents of 15 individual T2 seeds from a cotton embryoid carrying the *ghFAD2-1* intron-interrupted UTR inverted-repeat construct

T1 plant No.	T2 seed No.	Fatty acid composition (%)					ODP
		Palmitic	Stearic	Oleic	Linoleic	Linolenic	
10	1	24.1	2.1	57.8	15.8	0.1	0.22
	2	22.5	2.1	68.0	7.0	0.1	0.10
	3	23.0	2.3	67.5	6.9	0.2	0.09
	4	25.4	2.1	54.8	17.4	0.1	0.24
	5	22.8	2.6	27.9	46.3	0.2	0.63
	6	19.5	2.3	69.3	8.5	0.2	0.11
	7	19.9	2.2	70.6	6.9	0.1	0.09
	8	18.8	2.3	71.6	6.9	0.2	0.09
	9	20.2	2.1	70.1	7.2	0.1	0.10
	10	19.3	2.5	68.1	9.6	0.2	0.13
	11	19.0	2.0	68.8	9.7	0.1	0.13
	12	20.2	2.4	47.1	29.9	0.2	0.39
	13	18.3	2.1	70.4	8.6	0.2	0.11
	14	20.3	2.2	63.9	13.2	0.2	0.17
	15	18.5	2.5	68.7	9.8	0.1	0.13
	Mean	20.8	2.3	63.0	13.6	0.2	0.18

EXAMPLE 17

Characterisation of oils in transgenic seed that is homozygous for the transgene containing an inverted repeat of a fatty acid Δ12-desaturase gene

T2 Lines that are homozygous for the introduced inverted repeat of the cotton fatty acid Δ12-desaturase gene were identified by selection. The fatty acid compositions of individual T2 seeds (15 in total), derived from several

representative T1 plants that differed widely in their stearic acid content, were analysed as described in the preceding example. Data are presented in Table 13

It is clear that different transgenic events are associated with different levels of reduction in Δ12-desaturase activity (as indicated by ODP) and corresponding different levels of accumulation of the oleic acid substrate. This variation is explainable by different transgene copy numbers and different genomic integration locations of the transgenes. By producing a sufficient number of independent transgenics, any particular desired oleic acid content between the normal upper limit for cotton (approximately 16%) and approximately 75% can therefore be obtained (see Figure 12).

To confirm that the observed Δ12-desaturase gene silencing was heritable, additional T2 seed, and T3 seed, were obtained from plants exhibiting the most severe silencing phenotype, in particular, the independent T1 plants having the higher levels of oleic acid in their seed and designated 125-124 and 125-23 (Table 10). The fatty acid compositions of 15 individual T2 seed and 12 individual T3 seed from these lines were determined as described in the preceding example. The T2 segregation patterns were consistent with a single transgene insertion (Table 13). Data presented in Table 14 demonstrate that the high level silencing observed in the pooled T2 seeds of both lines as shown in Table 10, or the individual T2 seeds of line 125-23 as shown in Table 13, is inherited in the T3 generation, as evidenced by the heritability of the high oleic acid and low linoleic acid and low palmitic acid phenotypes.

TABLE 13

Fatty acid composition (% of total fatty acids) of 15 individual T2 seeds from five T1 plants of cotton transformed with the Δ12-desaturase inverted-repeat gene silencing construct and from untransformed Coker control plants.

T1 plant No.	T2 seed No.	Fatty acid composition (%)					ODP
		Palmitic	Stearic	Oleic	Linoleic	Linolenic	
Coker control	1	25.1	2.6	15.2	56.6	0.2	0.79
	2	25.4	2.8	15.7	55.6	0.2	0.78
	3	25.1	2.6	14.9	57.0	0.2	0.79
	4	23.9	2.7	15.1	58.0	0.2	0.79
	5	24.3	2.2	14.7	58.3	0.2	0.80
	6	25.2	2.5	14.4	57.4	0.2	0.80
	7	24.0	2.9	16.2	56.4	0.2	0.78
	8	24.9	2.3	15.0	57.4	0.2	0.79
	9	24.4	2.8	15.0	57.3	0.2	0.79
	10	23.3	3.0	14.7	58.5	0.2	0.80
	11	23.1	3.2	14.9	58.2	0.2	0.80
	12	23.9	2.9	14.9	57.8	0.2	0.80
	13	25.2	2.6	14.8	56.9	0.2	0.79
	14	22.9	2.9	14.4	59.3	0.2	0.80
	15	25.0	2.7	15.5	56.3	0.2	0.78
	Mean	24.4	2.7	15.0	57.4	0.2	0.79
125-120	1	26.9	2.1	13.5	57.1	0.2	0.81
	2	25.0	2.7	19.7	52.1	0.2	0.73
	3	24.9	2.1	19.8	52.8	0.2	0.73
	4	22.9	2.2	25.7	48.8	0.2	0.66
	5	24.4	2.5	25.3	47.3	0.2	0.65
	6	24.0	2.3	27.2	46.0	0.2	0.63
	7	24.2	2.0	28.4	45.0	0.2	0.61
	8	24.6	2.3	31.2	41.5	0.1	0.57
	9	22.9	2.2	33.8	40.7	0.2	0.55
	10	25.2	2.2	34.6	37.6	0.2	0.52
	11	21.7	2.5	42.6	32.8	0.2	0.44
	12	22.6	2.7	44.4	29.8	0.2	0.40
	13	22.6	2.2	46.1	28.6	0.2	0.38
	14	23.0	2.0	46.4	28.1	0.2	0.38
	15	22.5	2.3	51.6	23.1	0.2	0.31
	Mean	23.8	2.3	32.7	40.8	0.2	0.56

TABLE 13 continued.

T1 plant No.	T2 No.	Fatty acid composition (%)					ODP
		Palmitic	Stearic	Oleic	Linoleic	Linolenic	
125-130	1	24.9	2.7	14.6	57.3	0.2	0.80
	2	18.8	2.5	71.6	6.5	0.2	0.09
	3	17.4	2.4	73.8	5.8	0.2	0.08
	4	19.7	2.3	71.5	6.0	0.2	0.08
	5	19.6	2.3	72.2	5.5	0.2	0.07
	6	18.9	2.5	72.4	5.6	0.2	0.07
	7	17.6	2.7	73.6	5.5	0.2	0.07
	8	20.5	2.3	71.8	5.1	0.2	0.07
	9	19.4	2.7	72.1	5.5	0.1	0.07
	10	19.8	2.4	72.6	5.3	0.0	0.07
	11	18.0	2.3	74.8	4.4	0.2	0.06
	12	18.1	2.5	74.4	4.5	0.2	0.06
	13	16.7	2.6	75.3	4.9	0.2	0.06
	14	18.3	2.6	73.7	4.9	0.2	0.06
	15	23.6	1.9	69.4	4.7	0.1	0.06
	Mean	19.4	2.4	68.9	8.8	0.2	0.12
125-121	1	24.9	2.6	17.4	54.7	0.2	0.76
	2	24.7	2.5	17.3	55.1	0.2	0.76
	3	23.8	3.0	18.2	54.6	0.2	0.75
	4	26.1	2.9	22.1	48.4	0.3	0.69
	5	26.0	2.1	22.0	49.5	0.2	0.69
	6	25.0	2.8	25.0	46.7	0.2	0.65
	7	25.2	2.6	29.0	42.7	0.1	0.60
	8	23.2	2.5	34.0	40.0	0.2	0.54
	9	25.0	2.3	38.7	33.6	0.2	0.47
	10	21.3	2.9	45.6	29.7	0.1	0.40
	11	23.8	2.4	45.8	27.6	0.1	0.38
	12	21.0	2.6	51.2	24.8	0.2	0.33
	13	20.1	2.4	65.5	11.5	0.2	0.15
	14	20.0	2.3	70.0	7.3	0.1	0.10
	15	19.9	2.3	71.6	5.7	0.1	0.08
	Mean	23.3	2.5	38.2	35.4	0.2	0.49

TABLE 13 continued.

T1 plant No.	T2 seed No.	Fatty acid composition (%)					ODP
		Palmitic	Stearic	Oleic	Linoleic	Linolenic	
125-81	1	25.0	3.0	14.6	56.8	0.2	0.80
	2	26.6	2.7	15.7	54.5	0.2	0.78
	3	25.4	2.8	16.2	55.2	0.2	0.77
	4	21.2	2.8	68.2	7.2	0.2	0.10
	5	19.3	2.5	70.9	6.7	0.2	0.09
	6	19.8	2.7	70.0	7.0	0.2	0.09
	7	20.8	2.5	69.9	6.2	0.2	0.08
	8	21.5	2.6	69.3	6.0	0.2	0.08
	9	19.7	2.7	70.9	6.2	0.2	0.08
	10	19.7	2.7	71.1	6.0	0.2	0.08
	11	20.0	2.7	70.3	6.3	0.2	0.08
	12	21.0	2.5	70.1	5.9	0.2	0.08
	13	20.8	2.6	70.7	5.3	0.2	0.07
	14	19.5	2.7	72.2	5.0	0.2	0.07
	15	19.5	2.9	72.1	4.8	0.1	0.06
	Mean	21.3	2.7	59.5	15.9	0.2	0.22
125-23	1	23.5	2.4	13.7	60.1	0.1	0.81
	2	24.2	2.5	14.1	58.8	0.1	0.81
	3	23.9	2.5	14.4	58.9	0.1	0.80
	4	23.9	2.4	15.2	58.1	0.1	0.79
	5	25.9	2.4	15.5	55.9	0.1	0.78
	6	17.8	2.3	72.1	7.4	0.1	0.09
	7	17.2	2.5	73.5	6.5	0.1	0.08
	8	18.0	2.0	73.8	5.9	0.1	0.08
	9	20.7	2.3	70.8	5.8	0.1	0.08
	10	21.1	2.3	70.2	6.0	0.1	0.08
	11	18.5	2.5	72.1	6.5	0.2	0.08
	12	18.1	2.5	73.8	5.1	0.1	0.07
	13	17.8	2.4	74.2	5.2	0.1	0.07
	14	17.1	2.7	74.6	5.0	0.1	0.06
	15	17.6	2.4	74.7	4.9	0.1	0.06
	Mean	20.4	2.4	53.5	23.3	0.1	0.32

TABLE 14

Fatty acid composition (% of total fatty acids) of 12 individual T3 seeds from two independently derived T2 plants of cotton transformed with a Δ12-desaturase inverted-repeat gene silencing construct.

T1 plant	T3 seed	Fatty acid composition (%)					ODP
		Palmitic	Stearic	Oleic	Linoleic	Linolenic	
125-23	1	15.8	2.3	77.1	4.2	0.2	0.05
	2	15.9	2.6	76.5	4.3	0.2	0.05
	3	15.4	2.6	77.2	4.1	0.2	0.05
	4	15.3	2.5	77.3	4.3	0.2	0.05
	5	15.7	2.7	77.0	3.9	0.2	0.05
	6	15.5	2.6	77.0	4.3	0.2	0.05
	7	15.3	2.7	77.1	4.3	0.2	0.05
	8	15.5	2.8	76.9	4.2	0.2	0.05
	9	17.0	2.5	75.7	4.1	0.2	0.05
	10	16.7	2.7	76.0	4.1	0.2	0.05
	11	17.0	2.7	75.6	4.1	0.2	0.05
	12	17.4	2.6	75.3	4.1	0.2	0.05
	Mean	*16.0*	*2.6*	*76.6*	*4.2*	*0.2*	*0.05*
125-124	1	18.0	2.1	73.5	6.0	0.3	0.08
	2	20.8	2.1	71.1	5.5	0.3	0.07
	3	18.7	2.2	73.0	5.7	0.2	0.07
	4	18.2	2.2	72.4	6.9	0.2	0.09
	5	18.9	2.2	72.7	5.6	0.3	0.07
	6	17.8	2.2	72.7	6.8	0.3	0.09
	7	17.5	2.5	73.6	5.9	0.4	0.08
	8	21.1	2.1	70.8	5.7	0.2	0.08
	9	18.5	2.3	73.6	5.4	0.2	0.07
	10	18.5	2.2	72.4	6.5	0.3	0.08
	11	18.3	2.3	72.7	6.4	0.2	0.08
	12	18.5	2.3	73.1	5.7	0.3	0.07
	Mean	*18.7*	*2.2*	*72.6*	*6.0*	*0.3*	*0.08*

EXAMPLE 18

Sexual hybridisation of elite transgenic lines to enhance cotton seed oil characteristics

To produce a cotton seed oil which is rich in both oleic acid and stearic acid, and preferably low in palmitic acid, we conducted preliminary crosses between some of the transgenic T1 plant lines that produce high levels of stearic acid and oleic acid, as discussed in the preceding examples. In particular, line 95-150, which produces high stearic acid, and has reduced palmitic acid, oleic acid, and linoleic acid, was crossed to line 125-23, or line 125-96, or line 125-62, all of which produce high levels of oleic acid, and have low palmitic acid, and low linoleic acid content. We analysed the fatty acid composition of the F1 seed produced from those sexual hybridisations. Some of the novel fatty acid profiles detected in the F1 seed are summarised in Table 15.

Increased stearic acid

In the F1 seeds of three different crosses shown in Table 15, stearic acid content ranged from 7.1-14.9% of total seed lipid, compared to only 2.0% for non-transformed Coker cotton. Accordingly, these data indicate that it is possible to increase the level of stearic acid in cotton by as much as about 3.5-fold to about 7.5-fold, by expressing inverted repeats in cotton which target independently the endogenous cotton fatty acidΔ9-desaturase and Δ12-desaturase genes, and then crossing the transgenic plants.

Increased oleic acid

Oleic acid content ranged from 59.9% to 67.1% of total seed lipid in the F1 seed, compared to only 19.6% for non-transformed Coker cotton. Accordingly, these data indicate that it is possible to increase the level of oleic acid in cotton by up to about 3.5-fold, by expressing inverted repeats in cotton which target independently the endogenous cotton fatty acid Δ9-desaturase and Δ12-desaturase genes, and then crossing the transgenic plants.

Decreased linoleic acid

Linoleic acid content ranged from only 4.8% to 6.6% of total seed lipid in the F1 seed, compared to 54.4% for non-transformed Coker cotton. Accordingly, these data indicate that it is possible to decrease the level of linoleic acid in cotton by up to about 91.1%, by expressing inverted repeats in cotton which target independently the endogenous cotton fatty acid Δ9-desaturase and Δ12-desaturase genes, and then crossing the transgenic plants.

Decreased palmitic acid

Palmitic acid content ranged from only 17.6% to 19.5% of total seed lipid in the F1 seed, compared to 23.6% for non-transformed Coker cotton. Accordingly, these data indicate that it is possible to decrease the level of palmitic acid in cotton by up to about 25.4%, by expressing inverted repeats in cotton which target independently the endogenous cotton fatty acid Δ9-desaturase and Δ12-desaturase genes, and then crossing the transgenic plants.

TABLE 15

Average fatty acid compositions of F1 seed derived from sexual hybridisation of transgenic cotton lines

Cotton line	Average Fatty Acid Composition (%)					SDP	ODP
	Palmitic	Stearic	Oleic	Linoleic	Linolenic		
Coker	23.6	2.0	19.6	54.4	0.1	0.97	0.74
Transgenic parental lines							
125-23	16.8	1.4	77.0	4.4	0.2	0.98	0.06
125-62	17.6	2.3	66.1	13.5	0.3	0.97	0.17
125-96	19.4	2.2	74.1	3.8	0.2	0.97	0.05
95-150	19.0	28.0	5.8	45.4	0.3	0.65	0.89
F1 progeny of the cross 125-23 x 95-150							
F1 #1	18.5	8.6	65.8	6.0	0.1	0.89	0.09
F1 #2	17.9	12.1	62.1	6.6	0.2	0.85	0.10
F1 #3	17.6	12.5	63.0	5.7	0.1	0.85	0.08
F1 #4	17.9	10.0	65.5	5.5	0.1	0.88	0.08
F1 progeny of the cross 125-96 x 95-150							
F1 #1	19.5	7.1	67.6	4.8	0.1	0.91	0.07
F1 #2	19.2	7.1	66.9	5.9	0.1	0.91	0.08
F1 progeny of the cross 125-62 x 95-150							
F1#1	17.6	14.9	59.9	6.1	0.1	0.81	0.09

A population of 86 individual F2 seeds from the cross 125-23 x 95-150 was analysed for fatty acid composition as described in previous examples, to determine the range of fatty acid compositions obtained by recombination between the parental phenotypes. As shown in Figure 13, stearic acid and oleic acid compositions of cottonseed can be manipulated to produce levels of these fatty acids that range anywhere between the normal (wild type) level for untransformed Coker cotton and the highest levels present in the seed of transformed parental lines. Furthermore, the F2 seed comprise new cottonseed oils having different combinations of stearic acid and oleic acid content compared to untransformed cottonseed oil or individual transformed parental lines.

These results clearly demonstrated that the phenotypes observed for the transgenic parental lines are transmissible between generations, and can be combined in progeny plants. Accordingly, by selecting progeny plants which are homozygous for the introduced transgenes, and crossing those plants, elite lines containing elevated stearic acid and oleic acid, and having reduced levels of palmitic acid and linoleic acid, are obtained. Furthermore, crosses between elite lines which exhibit the highest levels of stearic acid and/or oleic acid, and the lowest levels of palmitic acid and/or linoleic acid, produces elite lines having the most desirable characteristics. Importantly, large scale intercrossing using parental lines produced as described herein, and having particular levels of individual fatty acids in their seed, is used to produce cotton seed having any desired level of palmitic acid, stearic acid, oleic acid, or linoleic acid, or a combination thereof, within the ranges observed in the parental lines. Accordingly, the present invention clearly provides a means by which cotton seed having the desirable POS fatty acid profile is produced, thereby providing a cotton seed oil substitute for cocoa butter.

EXAMPLE 19

Characterisation of oils in transgenic T2 seed containing antisense of a fatty acid Δ9 desaturase (stearoyl-ACP Δ9-desaturase) gene

Transgenic cotton having the fatty acid Δ9-desaturase antisense gene construct (Example 11) were analysed as described in the preceding examples for fatty acid composition. Data are presented in Table 16.

Increased stearic acid

Expression of the ghSAD-1 cDNA in the antisense orientation in cotton appeared to decrease the level of the stearoyl-ACP Δ9-desaturase enzyme activity in cotton, as suggested by the elevated level of stearic acid in transgenic T2 seed, particularly in line 9A-90. As summarised in Table 16, only one line of the total 25 transgenic lines generated produced seed having elevated stearic acid when compared to isogenic wild-type seed. Stearic acid was increased up to 28.2% of total seed lipid in line 9A-90 (Table 16).

Decreased oleic acid

Oleic acid was reduced in line 9A-90, from about 14.5% of total seed lipid in the non-transgenic cotton, to only 6.3% of total seed lipid.

Decreased linoleic acid

Linoleic acid was also reduced, from about 55% of total seed lipid in the non-transgenic line, to 46.2% in line 9A-90.

Decreased palmitic acid

Unexpectedly, and favorably, palmitic acid was significantly reduced in the T2 seed of transgenic cotton, particularly in line 9A-90. In particular, the level of palmitic acid in the seed of line 9A-90 was reduced to only 17.7% of total seed lipid in line 9A-90, compared to about 26% of total seed lipid for isogenic non-transformed plants.

TABLE 16

Fatty acid contents of T1 transgenic cotton comprising an antisense of a fatty acid Δ9-desaturase (stearoyl-ACP Δ9-desaturase) gene

Plant line	Fatty acid composition (%)					SDP
	Palmitic	Stearic	Oleic	Linoleic	Linolenic	
9A-118	26.7	2.0	15.8	55.2	0.1	0.97
9A-163	26.0	2.0	14.2	56.5	0.2	0.97
9A-38	27.0	2.2	14.0	56.4	0.3	0.97
9A-92	26.2	2.3	13.6	57.5	0.2	0.97
9A-9	25.2	2.3	13.6	58.5	0.1	0.97
9A-57	23.9	2.3	18.1	55.3	0.2	0.97
9A-127	24.2	2.4	15.2	57.8	0.2	0.97
9A-107	24.6	2.4	16.1	56.5	0.2	0.97
9A-148	24.2	2.4	15.1	57.9	0.1	0.97
9A-189	23.8	2.5	15.2	58.2	0.1	0.97
9A-89	23.8	2.5	15.7	57.5	0.2	0.97
9A-192	26.2	2.5	14.0	57.0	0.1	0.97
9A-14	28.5	2.4	14.9	53.9	0.1	0.97
9A-6	21.9	2.7	14.0	60.9	0.2	0.97
95-7	23.4	2.7	15.1	58.4	0.2	0.97
9A-2	23.6	2.5	14.0	59.6	0.1	0.97
9A-49	25.6	2.8	14.8	56.5	0.0	0.96
9A-81	24.2	2.8	14.4	58.1	0.2	0.96
9A-80	25.9	2.8	14.4	56.5	0.2	0.96
9A-150	22.9	3.2	15.4	58.1	0.1	0.96
9A-95	23.2	3.4	12.7	60.4	0.1	0.96
9A-183	24.3	3.4	14.2	57.6	0.2	0.96
9A-74	25.6	3.7	15.0	55.3	0.1	0.95
9A-28	21.8	5.4	12.4	60.0	0.1	0.93
9A-90	17.7	28.2	6.3	46.2	0.2	0.65

EXAMPLE 20

Characterisation of oils in transgenic T2 seed containing antisense of a fatty acid Δ12 desaturase (oleoyl-PC Δ12-desaturase) gene

Transgenic cotton having the fatty acid Δ12-desaturase antisense gene construct (Example 11) were analysed as described in the preceding examples for fatty acid composition. Data are presented in Table 17.

Increased oleic acid

In five (5) of the total twenty five (25) transgenic lines carrying the ghFAD2-1 cDNA in the antisense orientation, the level of oleic acid was increased to greater than 30% of total seed lipid (Table 17). The oleic acid content increased from about 14% for non-transformed Coker cotton, to as much as 74.4% of total seed lipid for the transgenic line 12A-87.

Decreased linoleic acid

Linoleic acid was reduced in transgenic cotton lines, from about 60% of total seed lipid in the non-transgenic Coker cotton, to only about 5.0% in line 12A-87.

Decreased palmitic acid

Unexpectedly, and favorably, palmitic acid was significantly reduced in the T2 seed of transgenic cotton containing the antisense gene construct. The level of palmitic acid in the seed of transgenic plants was reduced to only 18.3% of total seed lipid in line 12A-87, compared to about 26% of total seed lipid for isogenic non-transformed plants.

TABLE 17

Fatty acid contents of T1 transgenic cotton comprising an antisense of a fatty acid Δ12-desaturase (oleoyl-PC Δ12-desaturase) gene

Plant line	Fatty acid composition (%)					ODP
	Palmitic	Stearic	Oleic	Linoleic	Linolenic	
12A-30	23.4	2.6	13.2	60.3	0.2	0.82
12A-73	24.5	2.6	13.3	59.2	0.1	0.82
12A-124	24.7	2.8	13.7	58.4	0.2	0.81
12A-41	25.2	2.5	13.8	58.1	0.2	0.81
12A-60	22.9	2.6	14.4	59.6	0.2	0.81
12A-33	25.3	2.1	14.4	57.9	0.2	0.80
12A-111	26.1	2.5	14.2	56.7	0.2	0.80
12A-74	23.7	2.4	14.8	58.7	0.1	0.80
12A-84	24.0	2.6	14.9	58.0	0.2	0.80
12A-130	24.4	3.0	14.9	57.3	0.2	0.79
12A-59	24.6	2.2	15.1	57.7	0.2	0.79
12A-68	25.4	2.8	15.4	56.1	0.1	0.79
12A-85	27.8	2.4	16.2	53.1	0.2	0.77
12A-81	26.2	2.0	16.9	54.9	0.0	0.76
12A-48	22.2	2.5	17.8	57.3	0.1	0.76
12A-32	25.7	2.1	17.1	54.8	0.2	0.76
12A-132	24.7	2.2	21.4	51.3	0.1	0.71
12A-126	25.6	2.0	28.2	43.7	0.2	0.61
12A-1	21.1	3.5	29.7	45.2	0.2	0.60
12A-83	23.0	2.5	30.5	43.8	0.1	0.59
12A-118	23.9	2.0	34.5	39.6	0.0	0.53
12A-86	22.1	2.0	42.6	32.8	0.2	0.44
12A-14	19.7	2.1	72.4	5.4	0.1	0.07
12A-87	18.3	1.9	74.4	5.0	0.1	0.06

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WE CLAIM:

1. A method of modifying the endogenous oil of a cotton plant comprising producing a transgenic cotton plant having a gene construct which comprises a nucleotide sequence of a fatty acid biosynthesis gene or a gene fragment thereof operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant, and wherein said fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes, and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

2. The method according to claim 1 wherein the gene construct comprises a fatty acid biosynthesis gene or a gene fragment thereof which comprises a nucleotide sequence selected from the group consisting of:
   (i) the nucleotide sequence set forth in SEQ ID NO: 1 or a fragment thereof;
   (ii) a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO: 2 or a fragment thereof;
   (iii) a nucleotide sequence which is complementary to (i) or (ii); and
   (iv) an inverted repeat sequence having self-complementarity and consisting of a nucleotide sequence selected from the group consisting of:
      (a) the nucleotide sequence of (i) linked to the nucleotide sequence of (iii); and
      (b) the nucleotide sequence of (ii) linked to the nucleotide sequence of (iii).

3. The method according to claim 1 or 2 wherein the gene construct comprises the coding region of the nucleotide sequence set forth in SEQ ID NO: 1 or a fragment thereof in the antisense orientation.

4. The method according to claim 1 or 2 wherein the gene construct comprises an inverted repeat sequence of the 5'-terminal end of SEQ ID NO: 1 having self-complementarity.

5. The method according to claim 1 wherein the gene construct comprises a fatty acid biosynthesis gene or a gene fragment thereof which comprises a nucleotide sequence selected from the group consisting of:

(i) the nucleotide sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 7 or a fragment thereof;

(ii) a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO: 4 or a fragment thereof;

(iii) a nucleotide sequence which is complementary to (i) or (ii); and

(iv) an inverted repeat sequence having self-complementarity and consisting of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of (i) linked to the nucleotide sequence of (iii); and

(b) the nucleotide sequence of (ii) linked to the nucleotide sequence of (iii).

6. The method according to claim 1 or 5 wherein the gene construct comprises the coding region of the nucleotide sequence set forth in SEQ ID NO: 3 or a fragment thereof in the antisense orientation.

7. The method according to claim 1 or 5 wherein the gene construct comprises an inverted repeat sequence of the 5'-terminal end of SEQ ID NO: 3 having self-complementarity or an inverted repeat of the 5'-untranslated region of the *ghFAD2-1* gene set forth in SEQ ID NO: 7 having self-complementarity.

8. The method according to claim 1, wherein the promoter is the soybean lectin promoter sequence or the *ghFAD2-1* gene promoter.

9. The method according to any one of claims 1 to 8 wherein the gene construct is capable of being expressed in the cotton seed so as to reduce the level of expression of an endogenous cotton fatty acid biosynthesis gene to a level that is sufficient to modify the content and/or composition of the oil produced in said seed.

10. A transgenic cotton plant produced by the method according to any one of claims 1 to 9 and having a modified level of cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) in the seed compared to an isogenic non-transformed plant. or having a modified level of cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) in the seed compared to an isogenic non-transformed plant.

11. A cotton plant consisting of the progeny of the plant according to claim 10, wherein said progeny comprises the introduced fatty acid biosynthesis gene or gene fragment.

12. The cotton plant of claim 11 wherein said cotton plant has a modified level of cotton fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) in the seed compared to an isogenic non-transformed plant. or having a modified level of cotton fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) in the seed compared to an isogenic non-transformed plant.

13. A seed of the cotton plant according to any one of claims 10 to 12.

14. A method of modifying the endogenous oil of a cotton plant comprising:

(i) producing a transgenic cotton plant having a gene construct which comprises a nucleotide sequence of a fatty acid biosynthesis gene or a gene fragment thereof operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant; and

(ii) growing said plant for a time and under conditions sufficient for the expression of the corresponding endogenous fatty acid biosynthesis gene to be reduced in the seed by virtue of the presence of said nucleotide sequence in its genome,

wherein said fatty acid biosynthesis gene is selected from the group consisting of fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) genes and fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) genes.

15. The method according to claim 14 wherein the gene construct comprises a fatty acid biosynthesis gene or a gene fragment thereof which comprises a nucleotide sequence selected from the group consisting of:

(i) the nucleotide sequence set forth in SEQ ID NO: 1 or a fragment thereof;

(ii) a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO: 2 or a fragment thereof;

(iii) a nucleotide sequence which is complementary to (i) or (ii); and

(iv) an inverted repeat sequence having self-complementarity and consisting of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of (i) linked to the nucleotide sequence of (iii); and

(b) the nucleotide sequence of (ii) linked to the nucleotide sequence of (iii).

16. The method according to claim 14 or 15 wherein the gene construct comprises the coding region of the nucleotide sequence set forth in SEQ ID NO: 1 or a fragment thereof in the antisense orientation.

17. The method according to claim 14 or 15 wherein the gene construct comprises an inverted repeat sequence of the 5'-terminal end of SEQ ID NO:1 having self-complementarity.

18. A transgenic cotton plant produced by the method according to any one of claims 14 to 17 and having a reduced level of palmitic acid in the seed compared to an isogenic non-transformed plant.

19. A transgenic cotton plant produced by the method according to any one of claims 14 to 17 and having an increased level of stearic acid in the seed compared to an isogenic non-transformed plant.

20. A cotton plant consisting of the progeny of the plant according to claim 18, wherein said progeny has a decreased level of palmitic acid in the seed.

21. A cotton plant consisting of the progeny of the plant according to claim 19, wherein said progeny has an increased level of stearic acid in the seed.

22. A seed of the transgenic cotton plant according to claim 18 or 19.

23. A seed of the cotton plant according to claim 20 or 21.

24. The method according to claim 14 wherein the gene construct comprises a fatty acid biosynthesis gene or a gene fragment thereof which comprises a nucleotide sequence selected from the group consisting of:

(i) the nucleotide sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 7 or a fragment thereof;

(ii) a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO:4 or a fragment thereof;

(iii) a nucleotide sequence which is complementary to (i) or (ii); and

(iv) an inverted repeat sequence having self-complementarity and consisting of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of (i) linked to the nucleotide sequence of (iii); and

(b) the nucleotide sequence of (ii) linked to the nucleotide sequence of (iii).

25. The method according to claim 14 or 24 wherein the gene construct comprises the coding region of the nucleotide sequence set forth in SEQ ID NO: 3 or a fragment thereof in the antisense orientation.

26. The method according to claim 14 or 24 wherein the gene construct comprises an inverted repeat sequence of the 5'-terminal end of SEQ ID NO: 3 having self-complementarity or an inverted repeat of the 5'-untranslated region of the *ghFAD2-1* gene set forth in SEQ ID NO: 7 having self-complementarity.

27. A transgenic cotton plant produced by the method according to any one of claims 24 to 26 and having a reduced level of palmitic acid in the seed compared to an isogenic non-transformed plant.

28. A transgenic cotton plant produced by the method according to any one of claims 24 to 26 and having an increased level of oleic acid in the seed compared to an isogenic non-transformed plant.

29. A transgenic cotton plant produced by the method according to any one of claims 24 to 26 and having a decreased level of linoleic acid in the seed compared to an isogenic non-transformed plant.

30. A cotton plant consisting of the progeny of the plant according to claim 27, wherein said progeny has a decreased level of palmitic acid in the seed.

31. A cotton plant consisting of the progeny of the plant according to claim 28, wherein said progeny has an increased level of oleic acid in the seed.

32. A cotton plant consisting of the progeny of the plant according to claim 29, wherein said progeny has a decreased level of linoleic acid in the seed.

33. A seed of the plant according to claim 27 or 30.

34. A seed of the plant according to claim 28 or 31.

35. A seed of the plant according to claim 29 or 32.

36. The method according to any one of claims 15 to 17 further comprising transforming the transgenic cotton plant or a cell of said plant with a second gene construct which comprises a nucleotide sequence of a fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene or a gene fragment thereof placed operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant for a time and under conditions sufficient to decrease the expression of the corresponding endogenous fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene in the seed of said cotton plant.

37. The method according to any one of claims 24 to 26 further comprising transforming the transgenic cotton plant or a cell of said plant with a second gene construct which comprises a nucleotide sequence of a fatty acid Δ9-desaturase (Δ9-stearoyl-ACP desaturase) gene or a gene fragment thereof placed operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant for a time and under conditions sufficient to decrease the expression of the corresponding endogenous fatty acid Δ9-desaturase (Δ9-stearoyl-ACP desaturase) gene in the seed of said cotton plant.

38. A method of modifying the endogenous oil of a cotton plant comprising:

(i) producing a transgenic cotton plant having a gene construct that comprises a nucleotide sequence of a fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene or a gene fragment thereof operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant, and growing said plant for a time and under conditions sufficient for the expression of the endogenous fatty acid Δ9-desaturase (Δ9 stearoyl-ACP desaturase) gene in the seed to be reduced;

(ii) producing a transgenic cotton plant having a gene construct that comprises a nucleotide sequence of a fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene or a gene fragment thereof operably in connection with a promoter sequence capable of conferring expression of said gene or gene fragment in the seed of a cotton plant, and gorwing said plant for a time and under conditions sufficient forthe expression of the endogenous fatty acid Δ12-desaturase (Oleoyl-PC Δ12-desaturase) gene in the seed to be reduced;

(iii) crossing the cotton plant of (i) with the cotton plant of (ii);and

(iv) selecting the progeny of said crossing wherein said progeny have modified fatty acid content compared to a nontransformed isogenic cotton plant.

39. A cotton plant produced by the method according to claim 38 and having a reduced level of palmitic acid or linoleic acid in the seed compared to an isogenic non-transformed plant.

40. A seed of the plant according to claim 39.

41. A cotton plant produced by the method according to claim 38 and having an increased level of stearic acid or oleic acid in the seed compared to an isogenic non-transformed plant.

42. A cotton plant produced by the method according to claim 38 and having increased levels of stearic acid and oleic acid in the seed compared to an isogenic non-transformed plant.

43. A seed of the plant according to claim 41.

44. A seed of the plant according to claim 42.

45. The method according to claim 38 wherein the transgenic cotton plant at (i) or (ii) is a progeny of a primary transformed plant.

46. A cotton plant having increased oleic acid and/or stearic acid in the seed wherein said plant is produced by a process comprising sexual hybridisation between a first cotton plant having increased oleic acid in the seed compared to an isogenic non-transformed cotton plant and a second cotton plant having increased stearic acid in the seed compared to an isogenic non-transformed cotton plant, and wherein said first cotton plant or said second cotton plant is produced by the method according to claim 14.

47. The cotton plant according to claim 46 wherein said plant has decreased palmitic acid or linoleic acid in the seed.

48. A seed produced by the plant according to claim 46.

49. Cottonseed oil from a plant produced according to the method of claim 15, wherein said oil has a characteristic selected from the group consisting of:

(i) a high stearic acid content;

(ii) a reduced oleic acid content;

(iii) a reduced palmitic acid content; and

(iv) a reduced linoleic acid content.

50. Cottonseed oil from a plant produced according to the method of claim 24, wherein said oil has a characteristic selected from the group consisting of:

(i) a high oleic acid content;

(ii) a reduced palmitic acid content; and

(iii) a reduced linoleic acid content.

51. Cottonseed oil from a plant produced according to the method of claim 38, wherein said oil has a characteristic selected from the group consisting of:

(i) a high oleic acid content;

(ii) a high stearic acid content;

(iii) a high stearic acid and a high oleic acid content;

(iv) a reduced palmitic acid content; and

(v) a reduced linoleic acid content.

52. Cottonseed oil from the plant according to claim 46, wherein said oil has a characteristic selected from the group consisting of:

(i) a high oleic acid content;

(ii) a high stearic acid content;

(iii) a high stearic acid and a high oleic acid content;

(iv) a reduced palmitic acid content; and

(v) a reduced linoleic acid content.

53. A gene construct for modifying the fatty acid composition of cottonseed oil, comprising the nucleotide sequence of a plant fatty acid biosynthesis gene, or a gene fragment thereof, placed operably in connection with a promoter sequence that is operable in cotton seed, wherein said gene is selected from the group consisting of:

(i) the nucleotide sequence set forth in SEQ ID NO: 1 or a fragment thereof;

(ii) a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO: 2 or a fragment thereof;

(iii) the nucleotide sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 7 or a fragment thereof;

(iv) a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO: 4 or a fragment thereof;

(v) a nucleotide sequence which is complementary to any one of (i) to (iv);

(vi) an inverted repeat sequence having self-complementarity and consisting of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of (i) linked to the nucleotide sequence of (v);

(b) the nucleotide sequence of (ii) linked to the nucleotide sequence of (v);

(c) the nucleotide sequence of (iii) linked to the nucleotide sequence of (v); and

(d) the nucleotide sequence of (iv) linked to the nucleotide sequence of (v).

54. The gene construct according to claim 53 comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3 or a fragment thereof in the antisense orientation.

55. The gene construct according to claim 53 comprising an inverted repeat sequence selected from the group consisting of:

(i) the 5'-terminal end of SEQ ID NO: 1 having self-complementarity;

(ii) the 5'-terminal end of SEQ ID NO: 3 having self-complementarity; and

(iii) the 5'-untranslated region of the *ghFAD2-1* gene set forth in SEQ ID NO: 7 having self-complementarity.

56. The gene construct according to any one of claims 53 to 55 wherein the promoter sequence is a soybean lectin gene promoter sequence or the *ghFAD2-1* gene promoter

57. The method according to any one of claims 2, 5, 15, 24, or 53, wherein the inverted repeat is interrupted by an intervening sequence.

58. The method of claim 57, wherein the intervening sequence is an intron sequence.

59. The method of claim 58, wherein the intron in an excisable intron.

60. The method of claim 59, wherein the excisable intron is the first intron of the *ghFAD2-1* gene

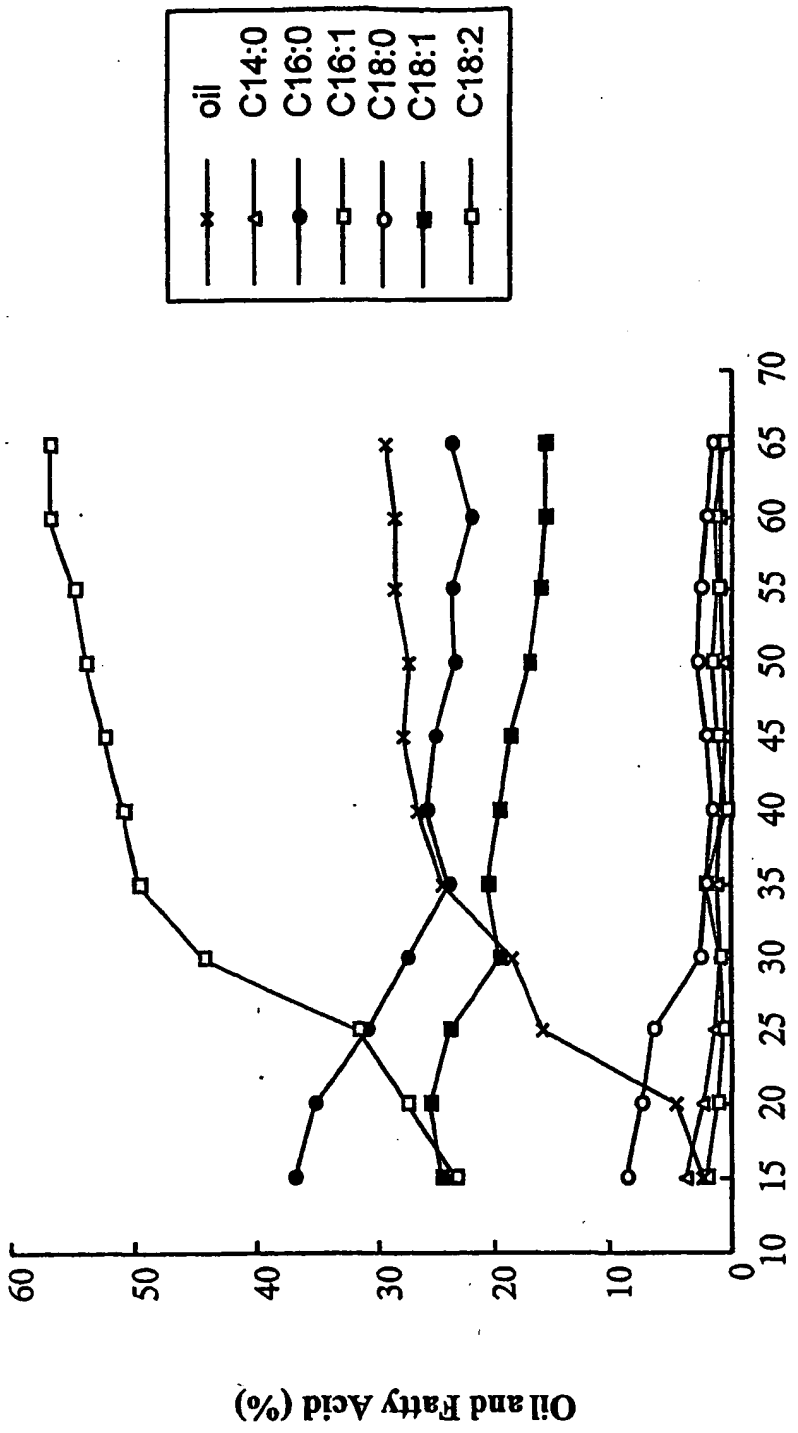
oil
C14:0
C16:0
C16:1
C18:0
C18:1
C18:2
Oil and Fatty Acid (%)
0
10
20
30
40
50
60
10
15
20
25
30
35
40
45
50
55
60
65
70
DAA (days)

FIGURE 1

```
CGAAAAGAAAAAATGGCTTTGAATTTTAATGCCATCGCCTCGAAATCTCAGAAGCTCCCT  60
            M  A  L  N  F  N  A  I  A  S  K  S  Q  K  L  P
TGCTTTGCTCTTCCACCAAAGGCCACCCTTAGATCTCCCAAGTTTTCCATGATCTCCACC 120
C  F  A  L  P  P  K  A  T  L  R  S  P  K  F  S  M  I  S  T
ATTCCTTCTGGCTCCAAAGAGGTTGGGAATCTGAAAAAGCCTTTCACGCCTCCAAAGGAG 180
I  P  S  G  S  K  E  V  G  N  L  K  K  P  F  T  P  P  K  E
GTGCCTGTTCAGATCACCCACTCCATGCCGCCTCACAAGATTGAGATCTTTAAATCTTTG 240
V  P  V  Q  I  T  H  S  M  P  P  H  K  I  E  I  F  K  S  L
GAGGGCTGGGCTGAGAACAACATTCTGACTCACCTCAAACCAGTTGAGAAATGTTGGCAA 300
E  G  W  A  E  N  N  I  L  T  H  L  K  P  V  E  K  C  W  Q
CCCGCCGACTTTCTTCCAGATCCTAATTCTGATGGATTTCATGAGCAAGTCAAAGAGCTT 360
P  A  D  F  L  P  D  P  N  S  D  G  F  H  E  Q  V  K  E  L
AGGGAAAGGGCAAAGGAGATCCCAGATGATTACTTTGTAGTTTTGGTTGGTGATATGATC 420
R  E  R  A  K  E  I  P  D  D  Y  F  V  V  L  V  G  D  M  I
ACCGAGGAAGCCCTTTCAACTTATCAAACAATGCTTAATACCTTGGATGGAACTCGTGAT 480
T  E  E  A  L  S  T  Y  Q  T  M  L  N  T  L  D  G  T  R  D
GAGACAGGTGCTAGCCTTACCCCTTGGGCCATTTGGACCAGGGCTTGGACTGCTGAAGAA 540
E  T  G  A  S  L  T  P  W  A  I  W  T  R  A  W  T  A  E  E
AACAGGCATGGTGATCTGCTTAATAAGTATCTCTACTTGTCTGGGAGAGTGGACATGAGG 600
N  R  H  G  D  L  L  N  K  Y  L  Y  L  S  G  R  V  D  M  R
CAAATTGAGAGGACAATCCAGTACTTGATTGGATCGGGAATGGATCCTCATACAGAGAAT 660
Q  I  E  R  T  I  Q  Y  L  I  G  S  G  M  D  P  H  T  E  N
AGTCCTTACCGAGGATTCATATATACTTCGTTCCAAGAAAGGGCAACTTTTATTTCCCAT 720
S  P  Y  R  G  F  I  Y  T  S  F  Q  E  R  A  T  F  I  S  H
GGGAATACAGGCAGGCTGGCTAAGGAGTATGGGGATATTAACTTGGCTCAAATTTGTGGT 780
G  N  T  G  R  L  A  K  E  Y  G  D  I  N  L  A  Q  I  C  G
AGCATTGCCTCAGATGAGAAGCGCCACGAGACAGCCTATACCAAAATCGTTGAAAAGCTG 840
S  I  A  S  D  E  K  R  H  E  T  A  Y  T  K  I  V  E  K  L
TTTGAGATTGATCCTGATGAAACAGTCCTGGCATTTGCTGACATGATGAAGAAGAAAATC 900
F  E  I  D  P  D  E  T  V  L  A  F  A  D  M  M  K  K  K  I
GCCATGCCGGCTGAGTTCATCTATGATGGCAGAGATTATAACTTATTTGACCACTACTCA 960
A  M  P  A  E  F  I  Y  D  G  R  D  Y  N  L  F  D  H  Y  S
GCTGTTGCCCAAAGAATCGGGGTTTACACTGCTAAGGACTATGTTGATATAGTAGAGCAC 1020
A  V  A  Q  R  I  G  V  Y  T  A  K  D  Y  V  D  I  V  E  H
CTGGTGGATCGATGGAAGGTGAAGGAGCTAGCTGGGCTTTCAGCCGAGGGGCGTAAAGCT 1080
L  V  D  R  W  K  V  K  E  L  A  G  L  S  A  E  G  R  K  A
CAGGACTACTTGTGTTCACTTCCTTCGAGAATTAGAAGGTTAGAGGAGAGAGCGCAAGAA 1140
Q  D  Y  L  C  S  L  P  S  R  I  R  R  L  E  E  R  A  Q  E
AAGGCCAAGGAAGCACCCAGTGTCCCATTCAGTTGGATATTTGATAGAGAAGTGAAACTT 1200
K  A  K  E  A  P  S  V  P  F  S  W  I  F  D  R  E  V  K  L
TAGGTCATGAAATACAGTTAAGACTCCTGCAATGCATTTGAGGAAACAAACACGAAGAAG 1260
*
CTGAATGCCAACTTCTCTTTATATATCCGATGTAATAGAGGTTGTATATGTAACAGGAGG 1320
AATTGCGTGGCTTTGGTTAGGGTAGCACATGTTTTCTGGATGTGTTGTGTCCTTAAAAAA 1380
TAATGCCGATAGCGGCAGCTGTGATAGTTTTAGATGTTTGTTTTCATAATGTCTGTTATA 1440
TCGTTGTACGAGTAGTATGTGTTGTTTTTGTTGAAACAATCTTCATATCTTAGTGATAAA 1500
TGATAATGCTGTGTAGTCATAGTTTTTAGTTTGCAATAAAAAAAAAAAAAAAA  1553
```

FIGURE 2

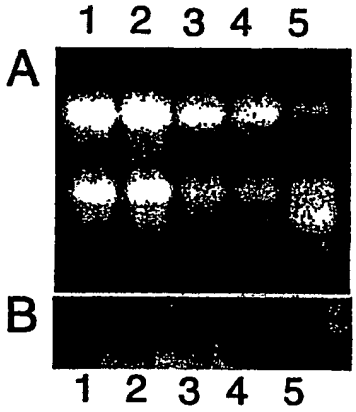

FIGURE 3

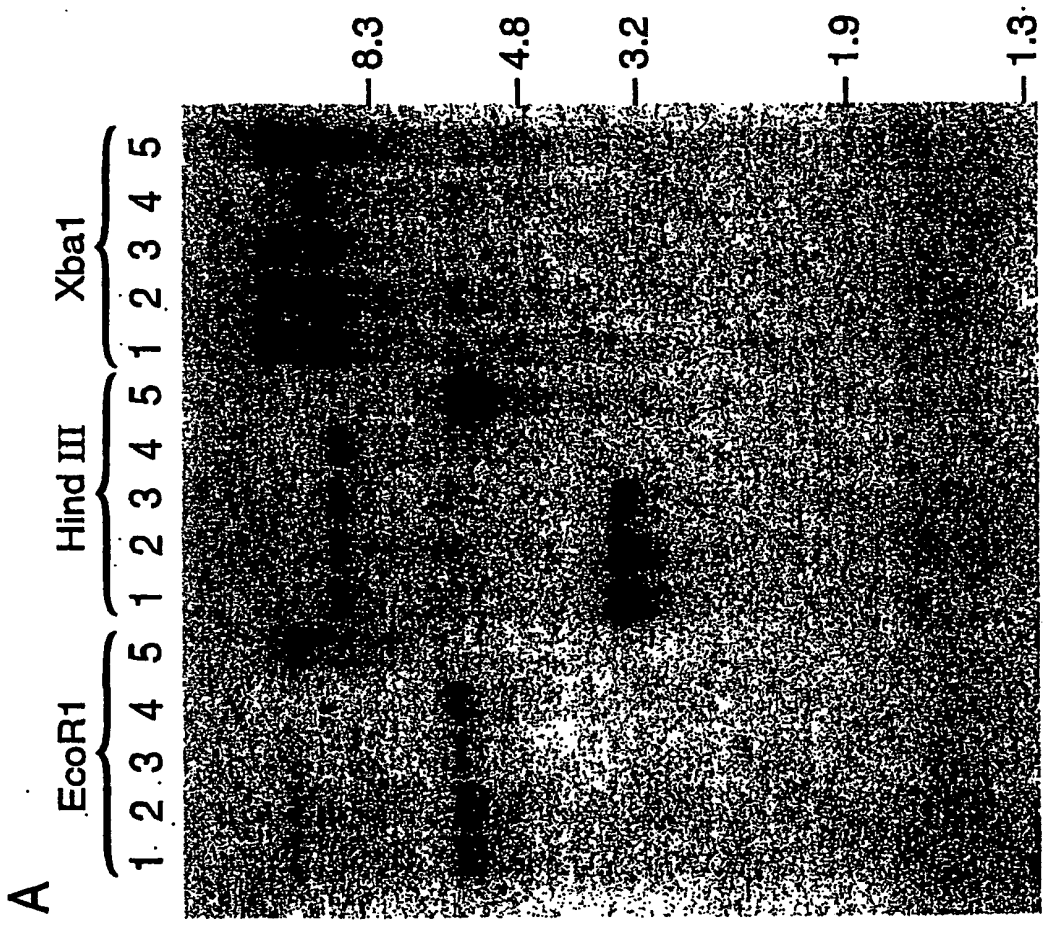

FIGURE 4A

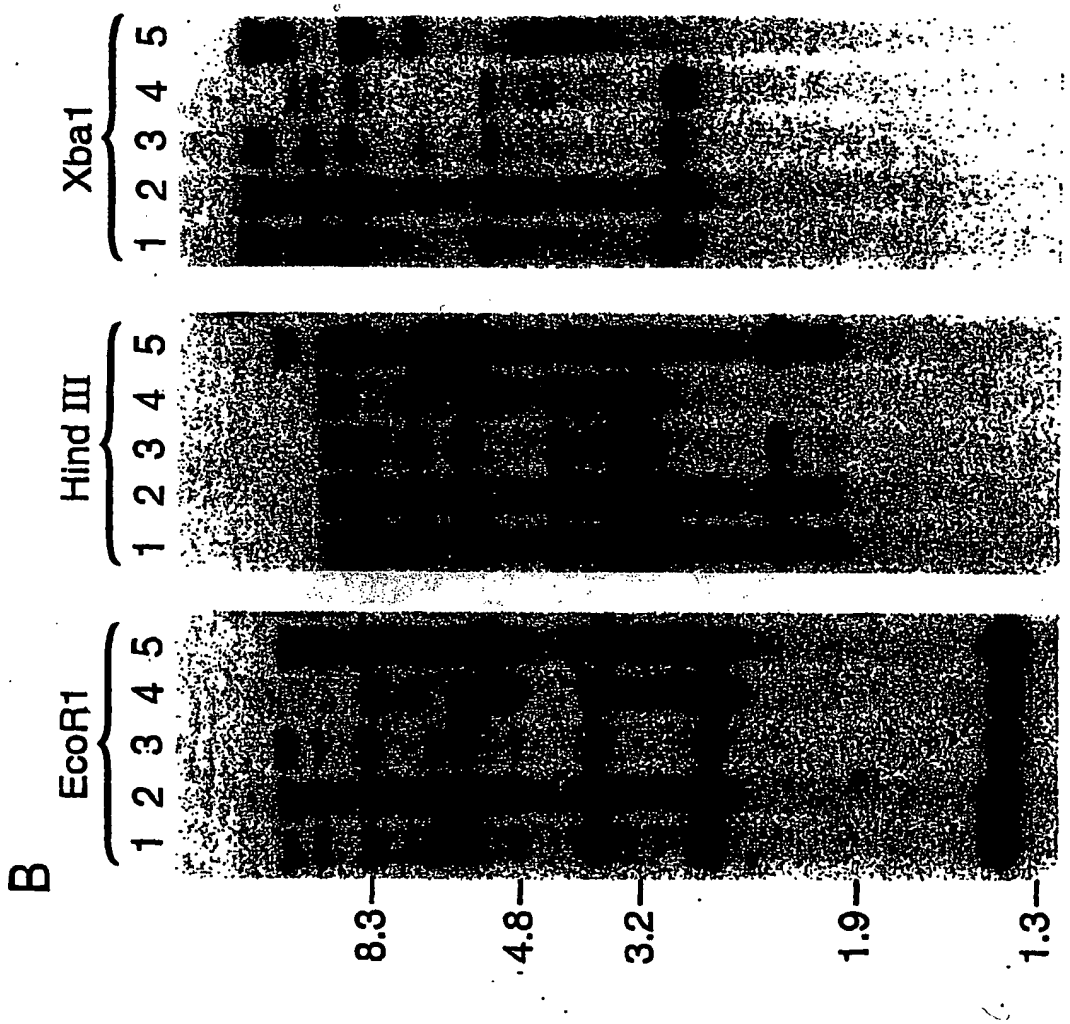

FIGURE 4B

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CTCGCCCAAAACCAACACGCCTTCTTTGCCTCGTGTTTCATCACCTGGCGTTAAACTGCT      60
TTCTTTAAAGCCAGCAAAATGGGTGCCGGTGGTAGGATGCCAATTGACGGTATAAAGGAG     120
                    M  G  A  G  G  R  M  P  I  D  G  I  K  E
GAAAATCGAGGCTCGGTCAATCGAGTTCCGATCGAGAAGCCTCCGTTTACGCTCGGTCAG     180
E  N  R  G  S  V  N  R  V  P  I  E  K  P  P  F  T  L  G  Q
ATCAAGCAAGCCATTCCGCCCCACTGTTTTCGCCGCTCCCTCCTTCGATCCTTCTCCTAC     240
I  K  Q  A  I  P  P  H  C  F  R  R  S  L  L  R  S  F  S  Y
GTGGTCCATGACCTATGCTTAGCCTCTTTCTTTTACTACATTGCAACATCATATTTTCAC     300
      d12A4 primer
V  V  H  D  L  C  L  A  S  F  F  Y  Y  I  A  T  S  Y  F  H
TTTCTCCCACAACCCTTTTCCTACATTGCTTGGCCTGTCTATTGGGTTCTCCAAGGTTGC     360
F  L  P  Q  P  F  S  Y  I  A  W  P  V  Y  W  V  L  Q  G  C
ATCCTCACCGGTGTTTGGGTCATCGCACACGAGTGGGGTCACCACGCTTTCAGAGACTAC     420
I  L  T  G  V  W  V  I  A  H  E  W  G  H  H  A  F  R  D  Y
CAATGGGTTGACGACACCGTCGGGTTGATCCTTCATTCCGCCCTTTTAGTCCCGTACTTC     480
Q  W  V  D  D  T  V  G  L  I  L  H  S  A  L  L  V  P  Y  F
TCGTGGAAAATCAGTCACCGCCGTCACCACTCGAACACCGGTTCCATGGAGCGTGACGAA     540
S  W  K  I  S  H  R  R  H  H  S  N  T  G  S  M  E  R  D  E
GTATTCGTGCCCAAACCCAAGTCTAAATTATCATGCTTTGCGAAATACTTAAACAATCCA     600
V  F  V  P  K  P  K  S  K  L  S  C  F  A  K  Y  L  N  N  P
CCCGGTCGAGTTCTATCTCTTGTAGTCACATTGACTCTTGGTTGGCCTATGTACTTAGCC     660
P  G  R  V  L  S  L  V  V  T  L  T  L  G  W  P  M  Y  L  A
TTCAACGTTTCGGGTCGATACTATGATCGATTAGCTTCCCACTATAACCCTTATGGCCCC     720
F  N  V  S  G  R  Y  Y  D  R  L  A  S  H  Y  N  P  Y  G  P
ATTTACTCCGATCGCGAGAGGCTACAAGTTTACATCTCCGATACTGGTATATTTGCGGTA     780
I  Y  S  D  R  E  R  L  Q  V  Y  I  S  D  T  G  I  F  A  V
ATTTATGTACTTTATAAGATTGCTGCAACAAAAGGGCTGGCTTGGCTTTTATGCACTTAT     840
I  Y  V  L  Y  K  I  A  A  T  K  G  L  A  W  L  L  C  T  Y
GGGGTGCCTCTACTTATTGTGAATGCCTTCCTTGTGTTGATCACCTACTTGCAACATACT     900
G  V  P  L  L  I  V  N  A  F  L  V  L  I  T  Y  L  Q  H  T
CACTCGGCATTGCCGCATTATGACTCGTCCGAATGGGATTGGTTGCGAGGAGCATTGTCG     960
H  S  A  L  P  H  Y  D  S  S  E  W  D  W  L  R  G  A  L  S
ACGATGGATCGAGATTTCGGGGTGTTGAACAAAGTGTTCCATAACATCACCGATACGCAT    1020
T  M  D  R  D  F  G  V  L  N  K  V  F  H  N  I  T  D  T  H
GTTGCTCATCACCTCTTCTCAACGATGCCACATTATCATGCAATGGAGGCCACTAAAGCA    1080
V  A  H  H  L  F  S  T  M  P  H  Y  H  A  M  E  A  T  K  A
ATCAAACCAATACTCGGCAAGTATTATCCTTTCGACGGGACACCGATTTACAAGGCAATG    1140
I  K  P  I  L  G  K  Y  Y  P  F  D  G  T  P  I  Y  K  A  M
TGGAGGGAGGCAAAAGAGTGCCTTTACGTTGAGCCTGACGTTGGTGGTGGTGGTGGTGGT    1200
W  R  E  A  K  E  C  L  Y  V  E  P  D  V  G  G  G  G  G  G
AGCAAAGGTGTTTTTTGGTATCGTAACAAGTTCTAAAGACCGACCAACTGCCTGATAGCT    1260
S  K  G  V  F  W  Y  R  N  K  F  *
GGCCGGCGAAATCAACGTAAAACGTACTTATTAGACTAGTGTTAACTAGGGAAGTTAATA    1320
ATTAATGGTAGGAAAATGTGGAATAGTTGCCTAGTAGTTTTATGTATTAAGTGTTGTATT    1380
AATAAACTATATGGTAGAAAAAAAAAAAAAA                                  1411
```

FIGURE 5

taaaaaaaaaaggcatttctttcatcttaaagagacagcgaggaagccacgaagataata
gagtgattttcaatctccattttaagggtgtggaacaatgggtgctggaggcagaatgtc
M G A G G R M S
ggttccaacgagtccaaaaaaacccgaattcaactcactgaagcgagttccatactcaaa
V P T S P K K P E F N S L K R V P Y S K
gccacccttcactctgagtgaaatcaagaaagccatcccaccacactgtttccagcgctc
P P F T L S E I K K A I P P H C F Q R S
cgttttacgctcattctcatatctcctttacgactttatattggcctctctttttttacca
V L R S F S Y L L Y D F I L A S L F Y H
tgtggccaccaattacttccctaaccttcctcaggctctctccaacgtggcttggcctct
V A T N Y F P N L P Q A L S N V A W P L
ttattgggccatgcaaggttgcattttgaccggcgtttgggtcatagcccatgaatgtgg
Y W A M Q G C I L T G V W V I A H E C G
ccaccatgctttcagtgattatcaatggcttgacgacaccgtgggccttatcctccactc
H H A F S D Y Q W L D D T V G L I L H S
ttctctcttagttccatatttctcttggaaatatagccaccggcgtcaccattctaacac
S L L V P Y F S W K Y S H R R H H S N T
cggttccctcgaaagggatgaagtgttcgttcccaagaaaaaatctggtttaagatggtg
G S L E R D E V F V P K K K S G L R W W
ggccaaacacttcaacaatccaccgggtcggtttctgtcaatcaccattcaacttaccct
A K H F N N P P G R F L S I T I Q L T L
tggttggccgctttacttagctttcaacgttgccggccggccttacgacaggttcgcttg
G W P L Y L A F N V A G R P Y D R F A C
ccactatgacccttacggccccatattttccgaccgggaacgactccaaatctatatctc
H Y D P Y G P I F S D R E R L Q I Y I S
tgacgccggcgtcctcgctgtcgcctatgcgctctaccgtctcgtgttggccaaaggggt
D A G V L A V A Y A L Y R L V L A K G V
aggttgggttattagcgtttatggggtgccattattggtggttaacgccttcttagtaat
G W V I S V Y G V P L L V V N A F L V M
gatcacgtatttgcaacacactcacccatctttgccgcactatgattcctcggagtggga
I T Y L Q H T H P S L P H Y D S S E W D
ctggatgagaggagctttatcaactgtggacagagattatgggatttaaacaaggtttt
W M R G A L S T V D R D Y G I L N K V F
ccataacataaccgacactcatgtggctcatcatttgttttcgacaatgcctcactatca
H N I T D T H V A H H L F S T M P H Y H
tgccatggtggccaccaaggcgataaagcccatattgggggaatactatcagttcgatgg
A M V A T K A I K P I L G E Y Y Q F D G
gatgcctgtctataaggcgatatggagggaggcgaaggagtgtctctacgttgaaccaga
M P V Y K A I W R E A K E C L Y V E P D
tgagggcgacaaggataaaggtgtgttttggtttagaaacaagctttaaatatttgcatt
E G D K D K G V F W F R N K L *
ttaccttaggcatgttctagtcgttgatgttttaaggatattttagccgacatacttggt
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FIGURE 6

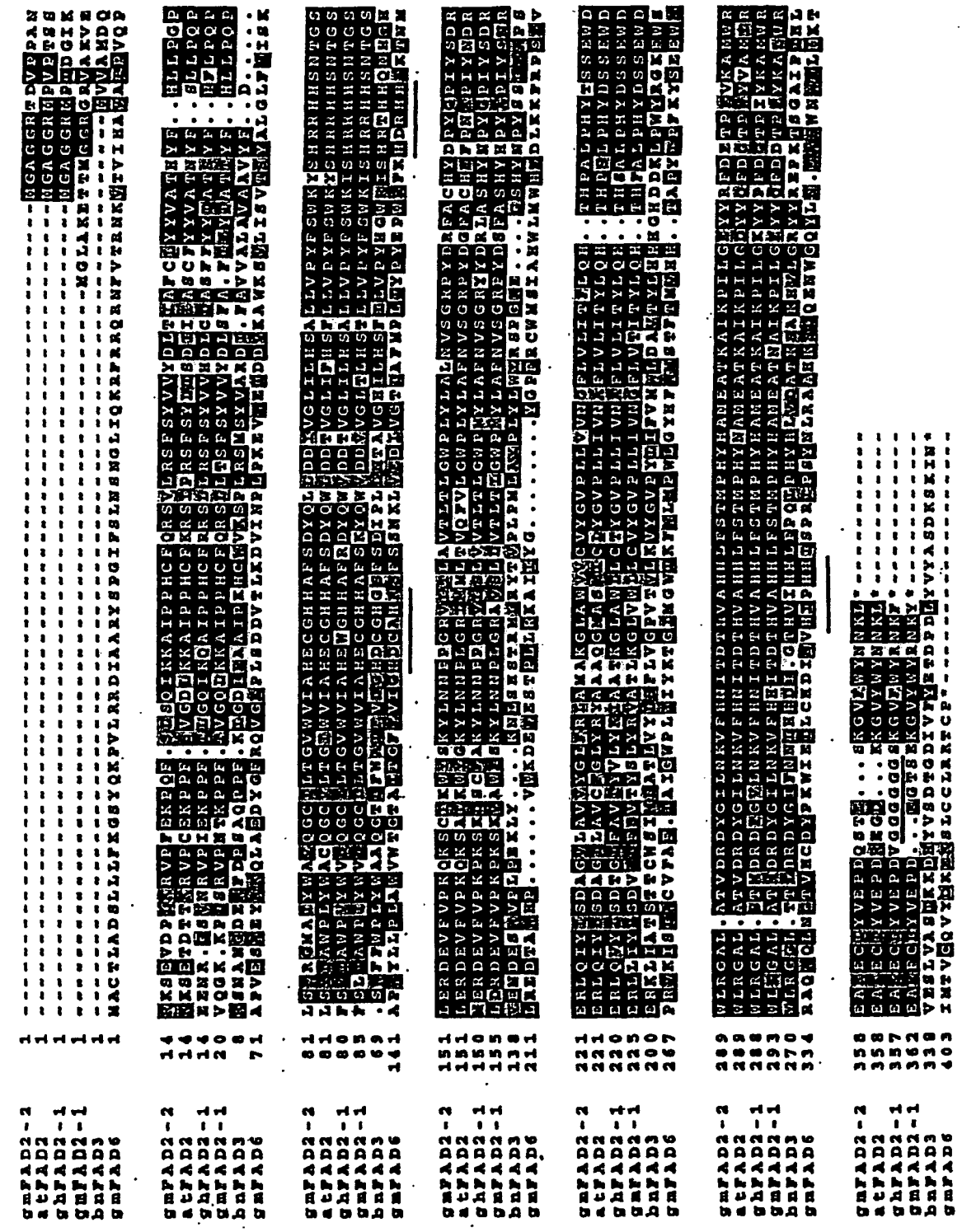


FIGURE 7

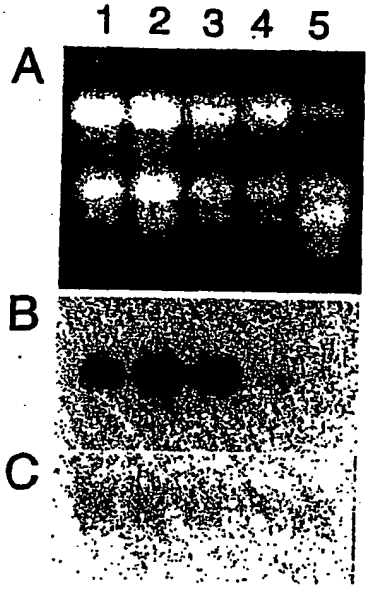


FIGURE 8

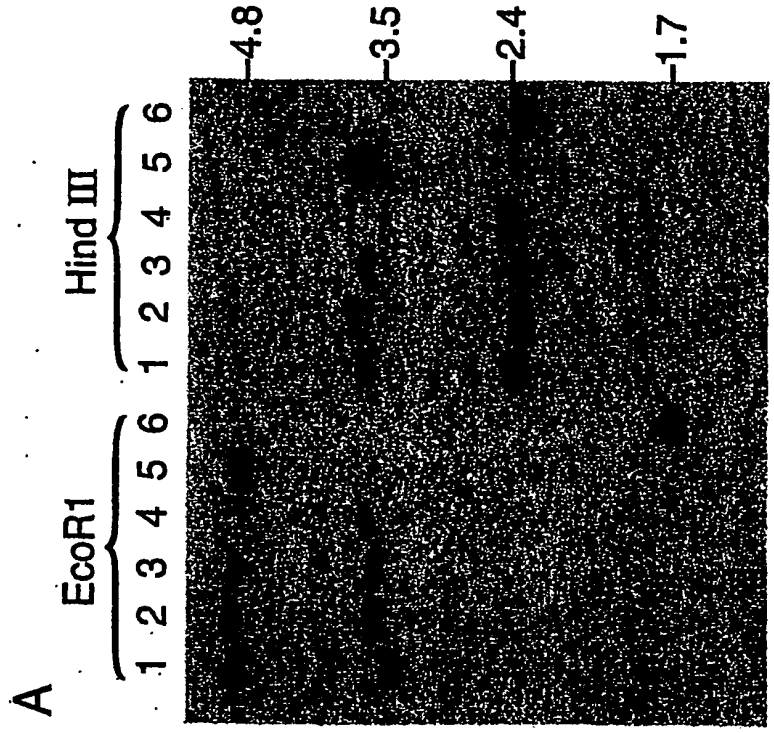


FIGURE 9A

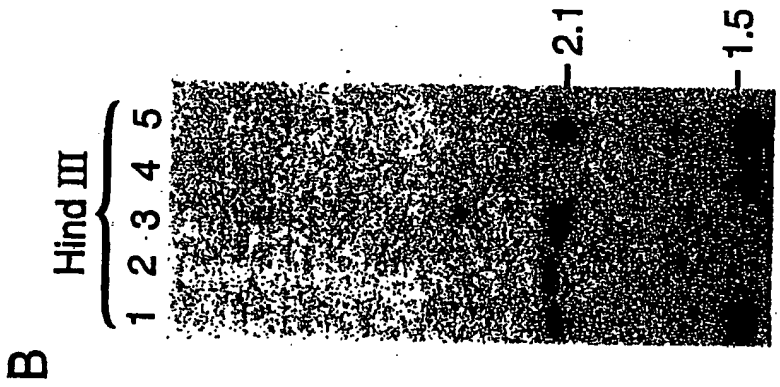


FIGURE 9B

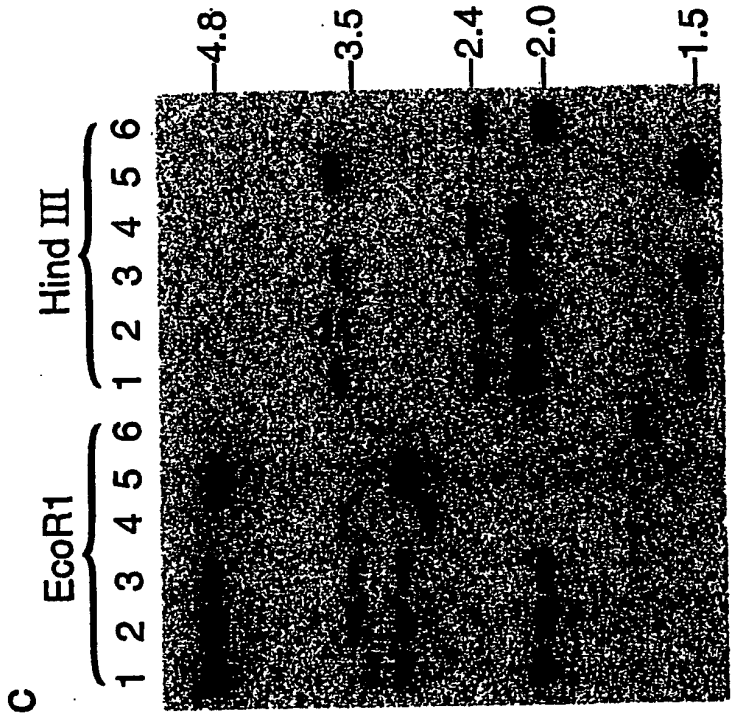
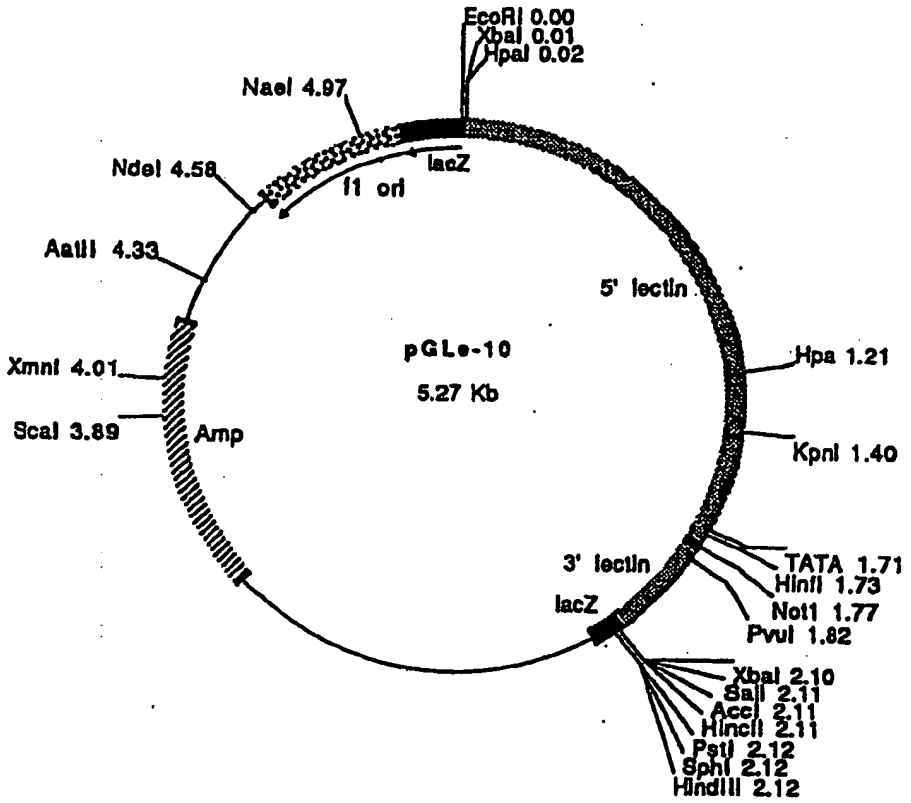


FIGURE 9C


EcoRI 0.00
XbaI 0.01
HpaI 0.02
NaeI 4.97
NdeI 4.58
AatII 4.33
XmnI 4.01
ScaI 3.89
lacZ
f1 ori
Amp
pGLe-10
5.27 Kb
5' lectin
3' lectin
lacZ
Hpa 1.21
KpnI 1.40
TATA 1.71
HinII 1.73
NotI 1.77
PvuI 1.82
XbaI 2.10
SalI 2.11
AccI 2.11
HincII 2.11
PstI 2.12
SphI 2.12
HindIII 2.12

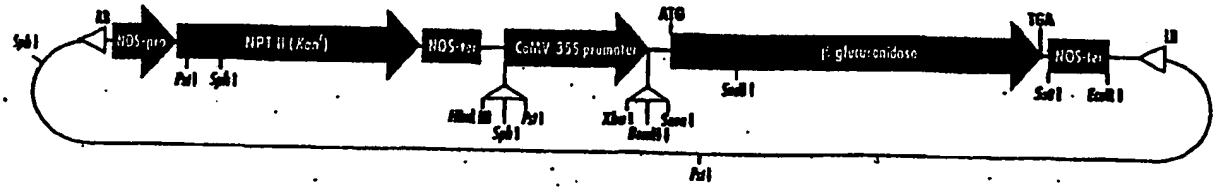
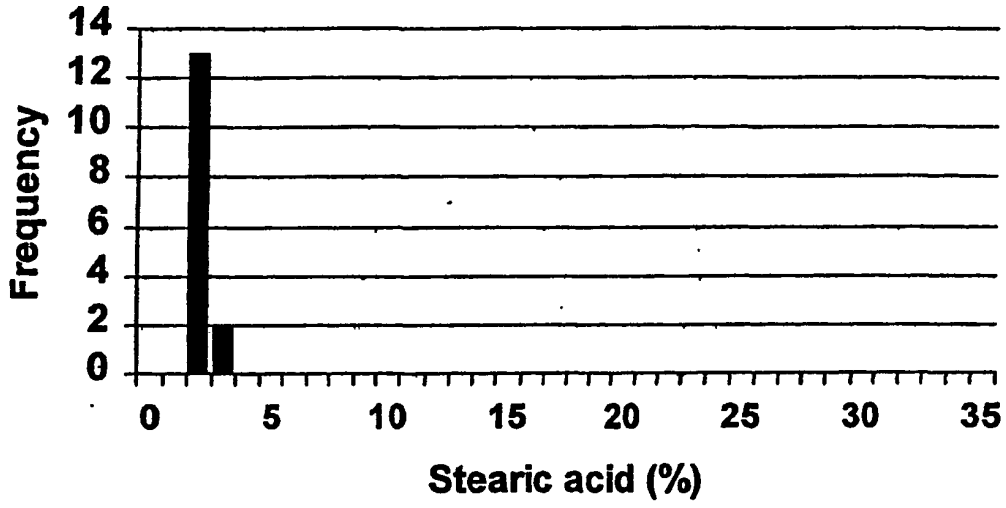

NPT II (Kan^r)
CaMV 35S promoter
β-glucuronidase
ATG
TGA

FIGURE 10


Frequency
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12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Stearic acid (%)

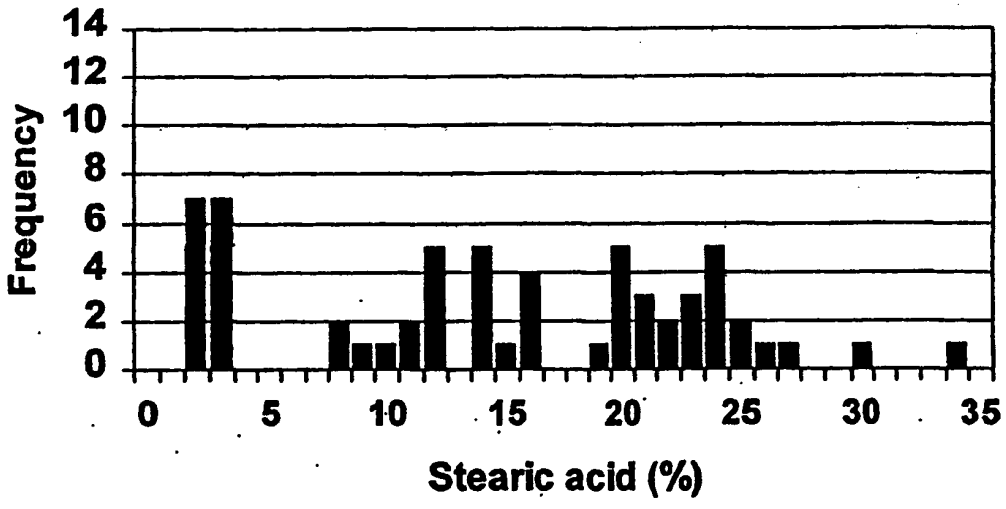
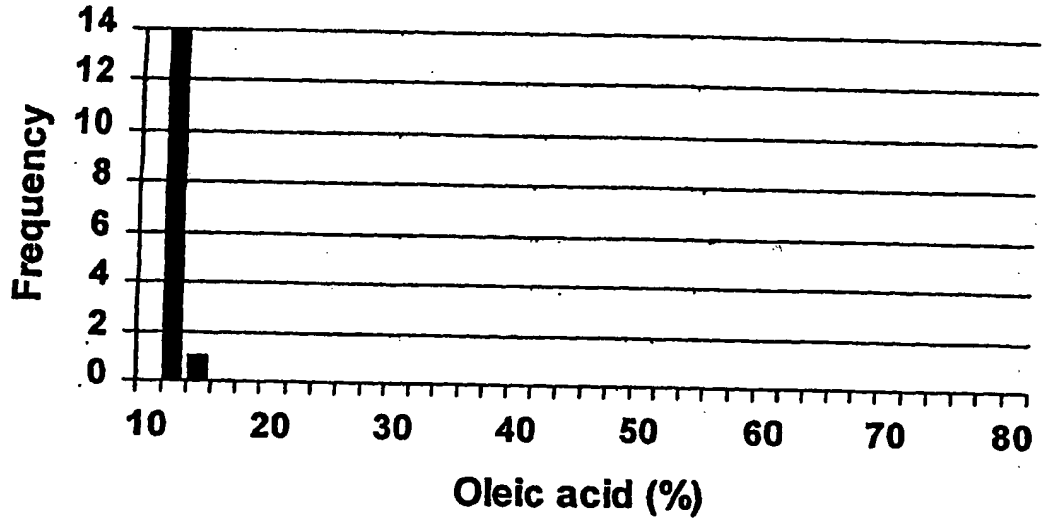

Frequency
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Stearic acid (%)

FIGURE 11


Frequency
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
Oleic acid (%)

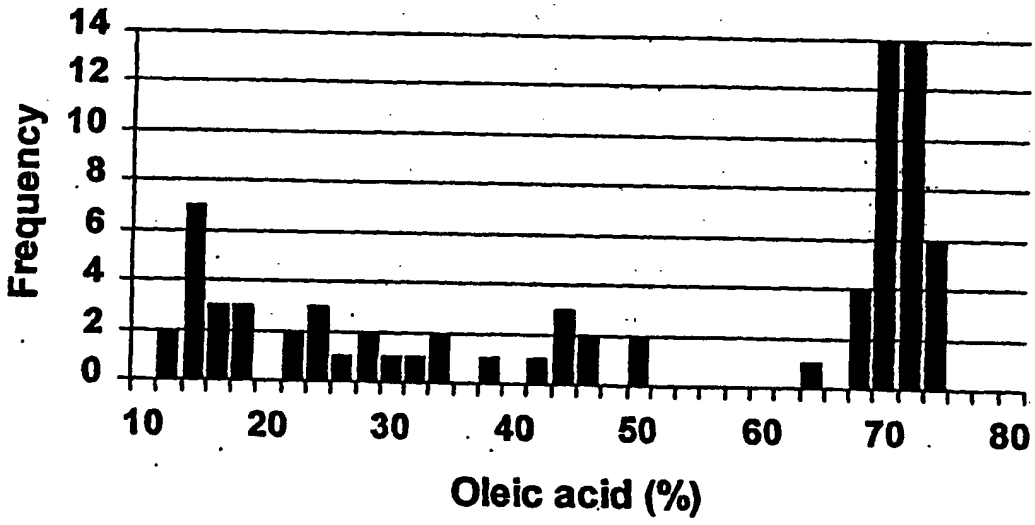

Frequency
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
Oleic acid (%)

FIGURE 12

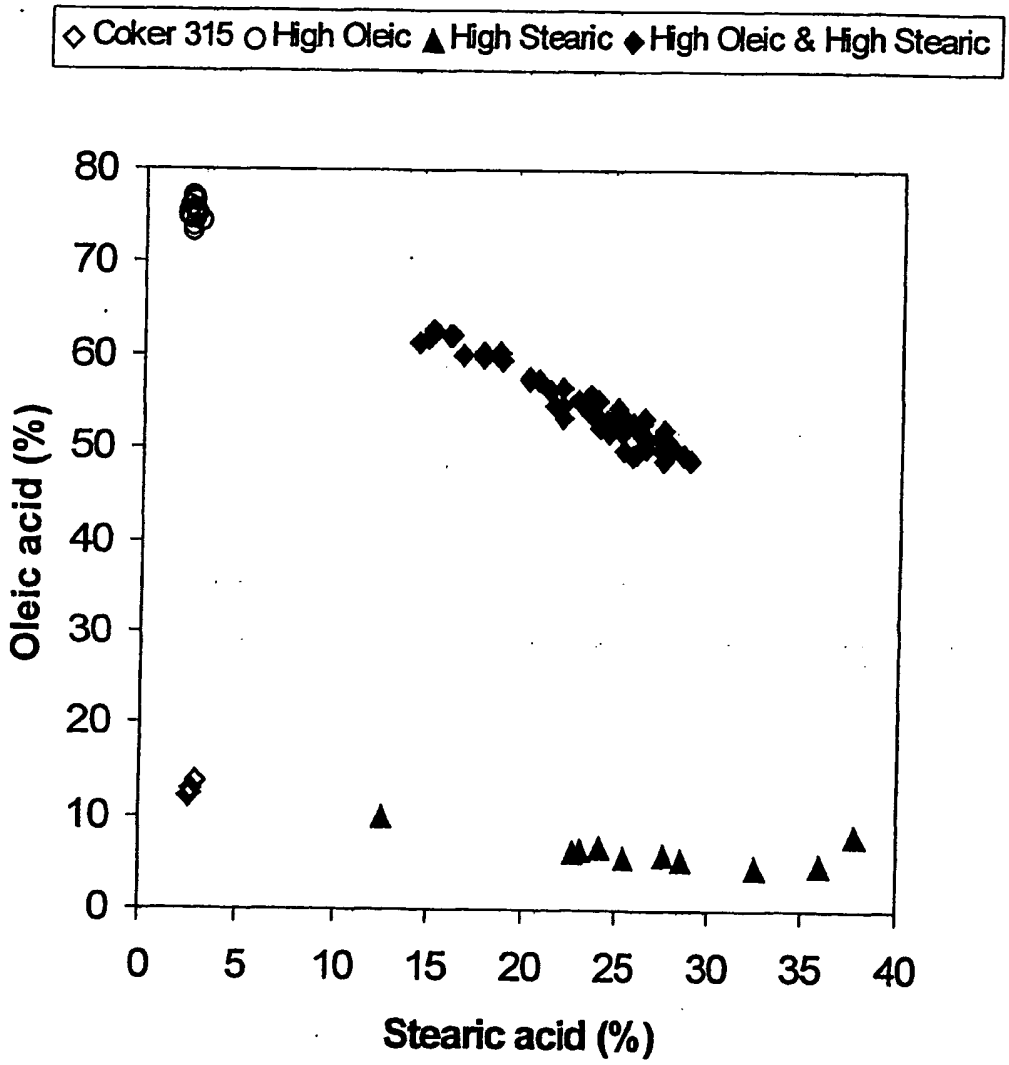

◇ Coker 315 ○ High Oleic ▲ High Stearic ◆ High Oleic & High Stearic
Oleic acid (%)
Stearic acid (%)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40

FIGURE 13

SEQUENCE LISTING

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<130> p:\oper\mro\specifications\cottonseed oil US

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<141> 2001-04-18

<150> US 60/198,124
<151> 2000-04-18

<160> 35

<170> PatentIn version 3.0

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aag ctc cct tgc ttt gct ctt cca cca aag gcc acc ctt aga tct ccc      99
Lys Leu Pro Cys Phe Ala Leu Pro Pro Lys Ala Thr Leu Arg Ser Pro
    15                  20                  25

aag ttt tcc atg atc tcc acc att cct tct ggc tcc aaa gag gtt ggg     147
Lys Phe Ser Met Ile Ser Thr Ile Pro Ser Gly Ser Lys Glu Val Gly
30                  35                  40                  45

aat ctg aaa aag cct ttc acg cct cca aag gag gtg cct gtt cag atc     195
Asn Leu Lys Lys Pro Phe Thr Pro Pro Lys Glu Val Pro Val Gln Ile
                50                  55                  60

acc cac tcc atg ccg cct cac aag att gag atc ttt aaa tct ttg gag     243
Thr His Ser Met Pro Pro His Lys Ile Glu Ile Phe Lys Ser Leu Glu
            65                  70                  75

ggc tgg gct gag aac aac att ctg act cac ctc aaa cca gtt gag aaa     291
Gly Trp Ala Glu Asn Asn Ile Leu Thr His Leu Lys Pro Val Glu Lys
        80                  85                  90

tgt tgg caa ccc gcc gac ttt ctt cca gat cct aat tct gat gga ttt     339
Cys Trp Gln Pro Ala Asp Phe Leu Pro Asp Pro Asn Ser Asp Gly Phe
    95                  100                 105

cat gag caa gtc aaa gag ctt agg gaa agg gca aag gag atc cca gat     387
His Glu Gln Val Lys Glu Leu Arg Glu Arg Ala Lys Glu Ile Pro Asp
110                 115                 120                 125

gat tac ttt gta gtt ttg gtt ggt gat atg atc acc gag gaa gcc ctt     435
Asp Tyr Phe Val Val Leu Val Gly Asp Met Ile Thr Glu Glu Ala Leu
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                    130                 135                 140

tca act tat caa aca atg ctt aat acc ttg gat gga act cgt gat gag      483
Ser Thr Tyr Gln Thr Met Leu Asn Thr Leu Asp Gly Thr Arg Asp Glu
            145                 150                 155

aca ggt gct agc ctt acc cct tgg gcc att tgg acc agg gct tgg act      531
Thr Gly Ala Ser Leu Thr Pro Trp Ala Ile Trp Thr Arg Ala Trp Thr
        160                 165                 170

gct gaa gaa aac agg cat ggt gat ctg ctt aat aag tat ctc tac ttg      579
Ala Glu Glu Asn Arg His Gly Asp Leu Leu Asn Lys Tyr Leu Tyr Leu
    175                 180                 185

tct ggg aga gtg gac atg agg caa att gag agg aca atc cag tac ttg      627
Ser Gly Arg Val Asp Met Arg Gln Ile Glu Arg Thr Ile Gln Tyr Leu
190                 195                 200                 205

att gga tcg gga atg gat cct cat aca gag aat agt cct tac cga gga      675
Ile Gly Ser Gly Met Asp Pro His Thr Glu Asn Ser Pro Tyr Arg Gly
                210                 215                 220

ttc ata tat act tcg ttc caa gaa agg gca act ttt att tcc cat ggg      723
Phe Ile Tyr Thr Ser Phe Gln Glu Arg Ala Thr Phe Ile Ser His Gly
            225                 230                 235

aat aca ggc agg ctg gct aag gag tat ggg gat att aac ttg gct caa      771
Asn Thr Gly Arg Leu Ala Lys Glu Tyr Gly Asp Ile Asn Leu Ala Gln
        240                 245                 250

att tgt ggt agc att gcc tca gat gag aag cgc cac gag aca gcc tat      819
Ile Cys Gly Ser Ile Ala Ser Asp Glu Lys Arg His Glu Thr Ala Tyr
    255                 260                 265

acc aaa atc gtt gaa aag ctg ttt gag att gat cct gat gaa aca gtc      867
Thr Lys Ile Val Glu Lys Leu Phe Glu Ile Asp Pro Asp Glu Thr Val
270                 275                 280                 285

ctg gca ttt gct gac atg atg aag aag aaa atc gcc atg ccg gct gag      915
Leu Ala Phe Ala Asp Met Met Lys Lys Lys Ile Ala Met Pro Ala Glu
                290                 295                 300

ttc atc tat gat ggc aga gat tat aac tta ttt gac cac tac tca gct      963
Phe Ile Tyr Asp Gly Arg Asp Tyr Asn Leu Phe Asp His Tyr Ser Ala
            305                 310                 315

gtt gcc caa aga atc ggg gtt tac act gct aag gac tat gtt gat ata     1011
Val Ala Gln Arg Ile Gly Val Tyr Thr Ala Lys Asp Tyr Val Asp Ile
        320                 325                 330

gta gag cac ctg gtg gat cga tgg aag gtg aag gag cta gct ggg ctt     1059
Val Glu His Leu Val Asp Arg Trp Lys Val Lys Glu Leu Ala Gly Leu
    335                 340                 345

tca gcc gag ggg cgt aaa gct cag gac tac ttg tgt tca ctt cct tcg     1107
Ser Ala Glu Gly Arg Lys Ala Gln Asp Tyr Leu Cys Ser Leu Pro Ser
350                 355                 360                 365

aga att aga agg tta gag gag aga gcg caa gaa aag gcc aag gaa gca     1155
Arg Ile Arg Arg Leu Glu Glu Arg Ala Gln Glu Lys Ala Lys Glu Ala
                370                 375                 380

ccc agt gtc cca ttc agt tgg ata ttt gat aga gaa gtg aaa ctt         1200
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Pro Ser Val Pro Phe Ser Trp Ile Phe Asp Arg Glu Val Lys Leu
385 390 395

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aattgcgtgg ctttggttag ggtagcacat gttttctgga tgtgttgtgt ccttaaaaaa 1320

taatgccgat agcggcagct gtgatagttt tagatgtttg ttttcataat gtctgttata 1380

tcgttgtacg agtagtatgt gttgtttttg ttgaaacaat cttcatatct tagtgataaa 1440

tgataatgct gtgtagtcat agtttttagt ttgcaataaa aaaaaaaaaa aaa 1493

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Cys Phe Ala Leu Pro Pro Lys Ala Thr Leu Arg Ser Pro Lys Phe Ser
20 25 30

Met Ile Ser Thr Ile Pro Ser Gly Ser Lys Glu Val Gly Asn Leu Lys
35 40 45

Lys Pro Phe Thr Pro Pro Lys Glu Val Pro Val Gln Ile Thr His Ser
50 55 60

Met Pro Pro His Lys Ile Glu Ile Phe Lys Ser Leu Glu Gly Trp Ala
65 70 75 80

Glu Asn Asn Ile Leu Thr His Leu Lys Pro Val Glu Lys Cys Trp Gln
85 90 95

Pro Ala Asp Phe Leu Pro Asp Pro Asn Ser Asp Gly Phe His Glu Gln
100 105 110

Val Lys Glu Leu Arg Glu Arg Ala Lys Glu Ile Pro Asp Asp Tyr Phe
115 120 125

Val Val Leu Val Gly Asp Met Ile Thr Glu Glu Ala Leu Ser Thr Tyr
130 135 140

Gln Thr Met Leu Asn Thr Leu Asp Gly Thr Arg Asp Glu Thr Gly Ala
145 150 155 160

Ser Leu Thr Pro Trp Ala Ile Trp Thr Arg Ala Trp Thr Ala Glu Glu
165 170 175

Asn Arg His Gly Asp Leu Leu Asn Lys Tyr Leu Tyr Leu Ser Gly Arg
180 185 190

Val Asp Met Arg Gln Ile Glu Arg Thr Ile Gln Tyr Leu Ile Gly Ser
195 200 205

Gly Met Asp Pro His Thr Glu Asn Ser Pro Tyr Arg Gly Phe Ile Tyr
210 215 220

Thr Ser Phe Gln Glu Arg Ala Thr Phe Ile Ser His Gly Asn Thr Gly
225 230 235 240

Arg Leu Ala Lys Glu Tyr Gly Asp Ile Asn Leu Ala Gln Ile Cys Gly
245 250 255

Ser Ile Ala Ser Asp Glu Lys Arg His Glu Thr Ala Tyr Thr Lys Ile
260 265 270

Val Glu Lys Leu Phe Glu Ile Asp Pro Asp Glu Thr Val Leu Ala Phe
275 280 285

Ala Asp Met Met Lys Lys Lys Ile Ala Met Pro Ala Glu Phe Ile Tyr
290 295 300

Asp Gly Arg Asp Tyr Asn Leu Phe Asp His Tyr Ser Ala Val Ala Gln
305 310 315 320

Arg Ile Gly Val Tyr Thr Ala Lys Asp Tyr Val Asp Ile Val Glu His
325 330 335

Leu Val Asp Arg Trp Lys Val Lys Glu Leu Ala Gly Leu Ser Ala Glu
340 345 350

Gly Arg Lys Ala Gln Asp Tyr Leu Cys Ser Leu Pro Ser Arg Ile Arg
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Arg Leu Glu Glu Arg Ala Gln Glu Lys Ala Lys Glu Ala Pro Ser Val
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Pro Phe Ser Trp Ile Phe Asp Arg Glu Val Lys Leu
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ttctttaaag ccagcaaa atg ggt gcc ggt ggt agg atg cca att gac ggt 111
Met Gly Ala Gly Gly Arg Met Pro Ile Asp Gly
1 5 10

ata aag gag gaa aat cga ggc tcg gtc aat cga gtt ccg atc gag aag 159
Ile Lys Glu Glu Asn Arg Gly Ser Val Asn Arg Val Pro Ile Glu Lys
15 20 25

cct ccg ttt acg ctc ggt cag atc aag caa gcc att ccg ccc cac tgt 207
Pro Pro Phe Thr Leu Gly Gln Ile Lys Gln Ala Ile Pro Pro His Cys
30 35 40

ttt cgc cgc tcc ctc ctt cga tcc ttc tcc tac gtg gtc cat gac cta 255
Phe Arg Arg Ser Leu Leu Arg Ser Phe Ser Tyr Val Val His Asp Leu
45 50 55

tgc tta gcc tct ttc ttt tac tac att gca aca tca tat ttt cac ttt 303
Cys Leu Ala Ser Phe Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Phe
60 65 70 75

ctc cca caa ccc ttt tcc tac att gct tgg cct gtc tat tgg gtt ctc 351
Leu Pro Gln Pro Phe Ser Tyr Ile Ala Trp Pro Val Tyr Trp Val Leu
80 85 90

caa ggt tgc atc ctc acc ggt gtt tgg gtc atc gca cac gag tgg ggt 399
Gln Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Trp Gly
95 100 105

cac cac gct ttc aga gac tac caa tgg gtt gac gac acc gtc ggg ttg 447
His His Ala Phe Arg Asp Tyr Gln Trp Val Asp Asp Thr Val Gly Leu
110 115 120

atc ctt cat tcc gcc ctt tta gtc ccg tac ttc tcg tgg aaa atc agt 495
Ile Leu His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser
125 130 135

cac cgc cgt cac cac tcg aac acc ggt tcc atg gag cgt gac gaa gta 543
His Arg Arg His His Ser Asn Thr Gly Ser Met Glu Arg Asp Glu Val
140 145 150 155

ttc gtg ccc aaa ccc aag tct aaa tta tca tgc ttt gcg aaa tac tta 591
Phe Val Pro Lys Pro Lys Ser Lys Leu Ser Cys Phe Ala Lys Tyr Leu
160 165 170

aac aat cca ccc ggt cga gtt cta tct ctt gta gtc aca ttg act ctt 639
Asn Asn Pro Pro Gly Arg Val Leu Ser Leu Val Val Thr Leu Thr Leu
175 180 185

ggt tgg cct atg tac tta gcc ttc aac gtt tcg ggt cga tac tat gat 687
Gly Trp Pro Met Tyr Leu Ala Phe Asn Val Ser Gly Arg Tyr Tyr Asp
190 195 200

cga tta gct tcc cac tat aac cct tat ggc ccc att tac tcc gat cgc 735
Arg Leu Ala Ser His Tyr Asn Pro Tyr Gly Pro Ile Tyr Ser Asp Arg
205 210 215

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gag agg cta caa gtt tac atc tcc gat act ggt ata ttt gcg gta att      783
Glu Arg Leu Gln Val Tyr Ile Ser Asp Thr Gly Ile Phe Ala Val Ile
220                 225                 230                 235

tat gta ctt tat aag att gct gca aca aaa ggg ctg gct tgg ctt tta      831
Tyr Val Leu Tyr Lys Ile Ala Ala Thr Lys Gly Leu Ala Trp Leu Leu
                240                 245                 250

tgc act tat ggg gtg cct cta ctt att gtg aat gcc ttc ctt gtg ttg      879
Cys Thr Tyr Gly Val Pro Leu Leu Ile Val Asn Ala Phe Leu Val Leu
            255                 260                 265

atc acc tac ttg caa cat act cac tcg gca ttg ccg cat tat gac tcg      927
Ile Thr Tyr Leu Gln His Thr His Ser Ala Leu Pro His Tyr Asp Ser
        270                 275                 280

tcc gaa tgg gat tgg ttg cga gga gca ttg tcg acg atg gat cga gat      975
Ser Glu Trp Asp Trp Leu Arg Gly Ala Leu Ser Thr Met Asp Arg Asp
    285                 290                 295

ttc ggg gtg ttg aac aaa gtg ttc cat aac atc acc gat acg cat gtt     1023
Phe Gly Val Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val
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gct cat cac ctc ttc tca acg atg cca cat tat cat gca atg gag gcc     1071
Ala His His Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala
                320                 325                 330

act aaa gca atc aaa cca ata ctc ggc aag tat tat cct ttc gac ggg     1119
Thr Lys Ala Ile Lys Pro Ile Leu Gly Lys Tyr Tyr Pro Phe Asp Gly
            335                 340                 345

aca ccg att tac aag gca atg tgg agg gag gca aaa gag tgc ctt tac     1167
Thr Pro Ile Tyr Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Leu Tyr
        350                 355                 360

gtt gag cct gac gtt ggt ggt ggt ggt ggt ggt agc aaa ggt gtt ttt     1215
Val Glu Pro Asp Val Gly Gly Gly Gly Gly Gly Ser Lys Gly Val Phe
    365                 370                 375

tgg tat cgt aac aag ttc taaagaccga ccaactgcct gatagctggc            1263
Trp Tyr Arg Asn Lys Phe
380                 385

cggcgaaatc aacgtaaaac gtacttatta gactagtgtt aactagggaa gttaataatt   1323

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Gly Gln Ile Lys Gln Ala Ile Pro Pro His Cys Phe Arg Arg Ser Leu
35 40 45

Leu Arg Ser Phe Ser Tyr Val Val His Asp Leu Cys Leu Ala Ser Phe
50 55 60

Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Phe Leu Pro Gln Pro Phe
65 70 75 80

Ser Tyr Ile Ala Trp Pro Val Tyr Trp Val Leu Gln Gly Cys Ile Leu
85 90 95

Thr Gly Val Trp Val Ile Ala His Glu Trp Gly His His Ala Phe Arg
100 105 110

Asp Tyr Gln Trp Val Asp Asp Thr Val Gly Leu Ile Leu His Ser Ala
115 120 125

Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His Arg Arg His His
130 135 140

Ser Asn Thr Gly Ser Met Glu Arg Asp Glu Val Phe Val Pro Lys Pro
145 150 155 160

Lys Ser Lys Leu Ser Cys Phe Ala Lys Tyr Leu Asn Asn Pro Pro Gly
165 170 175

Arg Val Leu Ser Leu Val Val Thr Leu Thr Leu Gly Trp Pro Met Tyr
180 185 190

Leu Ala Phe Asn Val Ser Gly Arg Tyr Tyr Asp Arg Leu Ala Ser His
195 200 205

Tyr Asn Pro Tyr Gly Pro Ile Tyr Ser Asp Arg Glu Arg Leu Gln Val
210 215 220

Tyr Ile Ser Asp Thr Gly Ile Phe Ala Val Ile Tyr Val Leu Tyr Lys
225 230 235 240

Ile Ala Ala Thr Lys Gly Leu Ala Trp Leu Leu Cys Thr Tyr Gly Val
245 250 255

Pro Leu Leu Ile Val Asn Ala Phe Leu Val Leu Ile Thr Tyr Leu Gln
260 265 270

His Thr His Ser Ala Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp
275 280 285

Leu Arg Gly Ala Leu Ser Thr Met Asp Arg Asp Phe Gly Val Leu Asn
290 295 300

Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His Leu Phe
305 310 315 320

Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala Ile Lys
325 330 335

Pro Ile Leu Gly Lys Tyr Tyr Pro Phe Asp Gly Thr Pro Ile Tyr Lys
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Ala Met Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Pro Asp Val
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Phe
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Met Gly Ala Gly Gly Arg
1 5

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Met Ser Val Pro Thr Ser Pro Lys Lys Pro Glu Phe Asn Ser Leu Lys
10 15 20

cga gtt cca tac tca aag cca ccc ttc act ctg agt gaa atc aag aaa 211
Arg Val Pro Tyr Ser Lys Pro Pro Phe Thr Leu Ser Glu Ile Lys Lys
25 30 35

gcc atc cca cca cac tgt ttc cag cgc tcc gtt tta cgc tca ttc tca 259
Ala Ile Pro Pro His Cys Phe Gln Arg Ser Val Leu Arg Ser Phe Ser
40 45 50

tat ctc ctt tac gac ttt ata ttg gcc tct ctt ttt tac cat gtg gcc 307
Tyr Leu Leu Tyr Asp Phe Ile Leu Ala Ser Leu Phe Tyr His Val Ala

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acc aat tac ttc cct aac ctt cct cag gct ctc tcc aac gtg gct tgg      355
Thr Asn Tyr Phe Pro Asn Leu Pro Gln Ala Leu Ser Asn Val Ala Trp
                75                  80                  85

cct ctt tat tgg gcc atg caa ggt tgc att ttg acc ggc gtt tgg gtc      403
Pro Leu Tyr Trp Ala Met Gln Gly Cys Ile Leu Thr Gly Val Trp Val
            90                  95                  100

ata gcc cat gaa tgt ggc cac cat gct ttc agt gat tat caa tgg ctt      451
Ile Ala His Glu Cys Gly His His Ala Phe Ser Asp Tyr Gln Trp Leu
        105                 110                 115

gac gac acc gtg ggc ctt atc ctc cac tct tct ctc tta gtt cca tat      499
Asp Asp Thr Val Gly Leu Ile Leu His Ser Ser Leu Leu Val Pro Tyr
    120                 125                 130

ttc tct tgg aaa tat agc cac cgg cgt cac cat tct aac acc ggt tcc      547
Phe Ser Trp Lys Tyr Ser His Arg Arg His His Ser Asn Thr Gly Ser
135                 140                 145                 150

ctc gaa agg gat gaa gtg ttc gtt ccc aag aaa aaa tct ggt tta aga      595
Leu Glu Arg Asp Glu Val Phe Val Pro Lys Lys Lys Ser Gly Leu Arg
                155                 160                 165

tgg tgg gcc aaa cac ttc aac aat cca ccg ggt cgg ttt ctg tca atc      643
Trp Trp Ala Lys His Phe Asn Asn Pro Pro Gly Arg Phe Leu Ser Ile
            170                 175                 180

acc att caa ctt acc ctt ggt tgg ccg ctt tac tta gct ttc aac gtt      691
Thr Ile Gln Leu Thr Leu Gly Trp Pro Leu Tyr Leu Ala Phe Asn Val
        185                 190                 195

gcc ggc cgg cct tac gac agg ttc gct tgc cac tat gac cct tac ggc      739
Ala Gly Arg Pro Tyr Asp Arg Phe Ala Cys His Tyr Asp Pro Tyr Gly
    200                 205                 210

ccc ata ttt tcc gac cgg gaa cga ctc caa atc tat atc tct gac gcc      787
Pro Ile Phe Ser Asp Arg Glu Arg Leu Gln Ile Tyr Ile Ser Asp Ala
215                 220                 225                 230

ggc gtc ctc gct gtc gcc tat gcg ctc tac cgt ctc gtg ttg gcc aaa      835
Gly Val Leu Ala Val Ala Tyr Ala Leu Tyr Arg Leu Val Leu Ala Lys
                235                 240                 245

ggg gta ggt tgg gtt att agc gtt tat ggg gtg cca tta ttg gtg gtt      883
Gly Val Gly Trp Val Ile Ser Val Tyr Gly Val Pro Leu Leu Val Val
            250                 255                 260

aac gcc ttc tta gta atg atc acg tat ttg caa cac act cac cca tct      931
Asn Ala Phe Leu Val Met Ile Thr Tyr Leu Gln His Thr His Pro Ser
        265                 270                 275

ttg ccg cac tat gat tcc tcg gag tgg gac tgg atg aga gga gct tta      979
Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp Met Arg Gly Ala Leu
    280                 285                 290

tca act gtg gac aga gat tat ggg att tta aac aag gtt ttc cat aac     1027
Ser Thr Val Asp Arg Asp Tyr Gly Ile Leu Asn Lys Val Phe His Asn
295                 300                 305                 310

ata acc gac act cat gtg gct cat cat ttg ttt tcg aca atg cct cac     1075
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Ile Thr Asp Thr His Val Ala His His Leu Phe Ser Thr Met Pro His
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tat cat gcc atg gtg gcc acc aag gcg ata aag ccc ata ttg ggg gaa     1123
Tyr His Ala Met Val Ala Thr Lys Ala Ile Lys Pro Ile Leu Gly Glu
            330                 335                 340

tac tat cag ttc gat ggg atg cct gtc tat aag gcg ata tgg agg gag     1171
Tyr Tyr Gln Phe Asp Gly Met Pro Val Tyr Lys Ala Ile Trp Arg Glu
        345                 350                 355

gcg aag gag tgt ctc tac gtt gaa cca gat gag ggc gac aag gat aaa     1219
Ala Lys Glu Cys Leu Tyr Val Glu Pro Asp Glu Gly Asp Lys Asp Lys
    360                 365                 370

ggt gtg ttt tgg ttt aga aac aag ctt taaatatttg cattttacct           1266
Gly Val Phe Trp Phe Arg Asn Lys Leu
375                 380

taggcatgtt ctagtcgttg atgttttaag gatattttag ccgacatact tggttttcct   1326

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Leu Ser Glu Ile Lys Lys Ala Ile Pro Pro His Cys Phe Gln Arg Ser
        35                  40                  45

Val Leu Arg Ser Phe Ser Tyr Leu Leu Tyr Asp Phe Ile Leu Ala Ser
    50                  55                  60

Leu Phe Tyr His Val Ala Thr Asn Tyr Phe Pro Asn Leu Pro Gln Ala
65                  70                  75                  80

Leu Ser Asn Val Ala Trp Pro Leu Tyr Trp Ala Met Gln Gly Cys Ile
                85                  90                  95

Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe
            100                 105                 110

Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Leu His Ser
        115                 120                 125
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Ser Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His
130 135 140

His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys
145 150 155 160

Lys Lys Ser Gly Leu Arg Trp Trp Ala Lys His Phe Asn Asn Pro Pro
165 170 175

Gly Arg Phe Leu Ser Ile Thr Ile Gln Leu Thr Leu Gly Trp Pro Leu
180 185 190

Tyr Leu Ala Phe Asn Val Ala Gly Arg Pro Tyr Asp Arg Phe Ala Cys
195 200 205

His Tyr Asp Pro Tyr Gly Pro Ile Phe Ser Asp Arg Glu Arg Leu Gln
210 215 220

Ile Tyr Ile Ser Asp Ala Gly Val Leu Ala Val Ala Tyr Ala Leu Tyr
225 230 235 240

Arg Leu Val Leu Ala Lys Gly Val Gly Trp Val Ile Ser Val Tyr Gly
245 250 255

Val Pro Leu Leu Val Val Asn Ala Phe Leu Val Met Ile Thr Tyr Leu
260 265 270

Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp Asp
275 280 285

Trp Met Arg Gly Ala Leu Ser Thr Val Asp Arg Asp Tyr Gly Ile Leu
290 295 300

Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His Leu
305 310 315 320

Phe Ser Thr Met Pro His Tyr His Ala Met Val Ala Thr Lys Ala Ile
325 330 335

Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Phe Asp Gly Met Pro Val Tyr
340 345 350

Lys Ala Ile Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Pro Asp
355 360 365

Glu Gly Asp Lys Asp Lys Gly Val Phe Trp Phe Arg Asn Lys Leu

370 375 380

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aaaggcccaa ctaatccaaa caaaacttga gtgttacaat ctaaccctag tctggcaacg 180

gatacgggtt aagggtgtta caaccttiac agtgatcaac gaacaaacct tgagtggatt 240

tggatttgac ccccctaccc cactacacac aaggaagaat gttagtttag ttattcaata 300

gctactaagt tggtttacat atatatacaa gttccacact tgattctcaa tcaatgtgag 360

actaatgctt ttcatttctc tcaacataat tcacaagtag cttactttga gtatcaattt 420

ttcattcatc actcaatcat tttgagcata tgatatattg ttgtaaatgt ctaatggagt 480

agaatataaa attataattt tatgattcaa cttttcacct ttaccaatag aaaatatgcc 540

tcaaagtttt caaaaaatca tatttttttt aatagaaata actttagaca tcaagaatct 600

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aaattcttct acttgagatt ttgatgcatg cgacaaaaat tgagattaga atccatgatg 720

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catacagcaa gcgatacttc caatgaccca aaattattga aaccaatgtg cttttcctat 960

cattgaaaat cgtgatggag ccacttgtaa cattgtttgc gtacatactt attattatta 1020

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tagaaatага tgaaatttтc aataaaaaag gaccagttta ctctttgaac taacgcacaa 1200

tgactaattt accattttta gtagatgagg taaaatataa tctagctcct tgtacagggg 1260

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gaaaattttg tttccaaaga taattacaaa gttaagtcaa acaagcagta acattgtttc 1380

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cacgtggcgt ctttatgtaa aaagaaatat tttgtttaat taaatgtata tacacattaa 1920

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caaaaatacа ataattgaaa aaaaattagt tgaaattaat aatattatta aaaaatgtaa 2040

aacatttgta aaaattataa aaaagtttta aaaataattt tctttataaa attctaaaat 2100

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taaatacttt ttcaattttt ataacacttt tttaattttt atatattatt ttggattttt 2220

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His Glu Xaa Gly His His
1 5

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<400> 27
His Arg Arg His His
1 5

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His Val Ala His His
1 5

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<220>
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<222> ()..()
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<400> 29

His Xaa Xaa His His
1 5

<210> 30
<211> 6
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<220>
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His Xaa Xaa Xaa His His
1 5

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<400> 31

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Glu Val Asp Pro Leu Lys Arg Val Pro Phe Glu Lys Pro Gln Phe Ser
20 25 30

Leu Ser Gln Ile Lys Lys Ala Ile Pro Pro His Cys Phe Gln Arg Ser
35 40 45

Val Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Thr Ile Ala Phe
50 55 60

Cys Leu Tyr Tyr Val Ala Thr His Tyr Phe His Leu Leu Pro Gly Pro
65 70 75 80

Leu Ser Phe Arg Gly Met Ala Ile Tyr Trp Ala Val Gln Gly Cys Ile
85 90 95

Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe
100 105 110

Ser Asp Tyr Gln Leu Leu Asp Asp Ile Val Gly Leu Ile Leu His Ser
115 120 125

Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His
130 135 140

His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys
145 150 155 160

Gln Lys Ser Cys Ile Lys Trp Tyr Ser Lys Tyr Leu Asn Asn Pro Pro
165 170 175

Gly Arg Val Leu Thr Leu Ala Val Thr Leu Thr Leu Gly Trp Pro Leu
180 185 190

Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys
195 200 205

His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asp Arg Glu Arg Leu Gln
210 215 220

Ile Tyr Ile Ser Asp Ala Gly Val Leu Ala Val Val Tyr Gly Leu Phe
225 230 235 240

Arg Leu Ala Met Ala Lys Gly Leu Ala Trp Val Val Cys Val Tyr Gly
245 250 255

Val Pro Leu Leu Val Val Asn Gly Phe Leu Val Leu Ile Thr Phe Leu
260 265 270

Gln His Thr His Pro Ala Leu Pro His Tyr Thr Ser Ser Glu Trp Asp
275 280 285

Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile Leu
290 295 300

Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His Leu
305 310 315 320

Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala Ile
325 330 335

Lys Pro Ile Leu Gly Glu Tyr Tyr Arg Phe Asp Glu Thr Pro Phe Val
340 345 350

Lys Ala Met Trp Arg Glu Ala Arg Glu Cys Ile Tyr Val Glu Pro Asp
355 360 365

Gln Ser Thr Glu Ser Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu
370 375 380

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Val Gly Asp Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser
35 40 45

Ile Pro Arg Ser Phe Ser Tyr Leu Ile Ser Asp Ile Ile Ile Ala Ser
50 55 60

Cys Phe Tyr Tyr Val Ala Thr Asn Tyr Phe Ser Leu Leu Pro Gln Pro
65 70 75 80

Leu Ser Tyr Leu Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val
85 90 95

Leu Thr Gly Ile Trp Val Ile Ala His Glu Cys Gly His His Ala Phe
100 105 110

Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser
115 120 125

Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His
130 135 140

His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys
145 150 155 160

Gln Lys Ser Ala Ile Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu
165 170 175

Gly Arg Ile Met Met Leu Thr Val Gln Phe Val Leu Gly Trp Pro Leu
180 185 190

Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Phe Ala Cys
195 200 205

His Phe Phe Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu Gln
210 215 220

Ile Tyr Leu Ser Asp Ala Gly Ile Leu Ala Val Cys Phe Gly Leu Tyr
225 230 235 240

Arg Tyr Ala Ala Ala Gln Gly Met Ala Ser Met Ile Cys Leu Tyr Gly
245 250 255

Val Pro Leu Leu Ile Val Asn Ala Phe Leu Val Leu Ile Thr Tyr Leu
260 265 270

Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp Asp
275 280 285

Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile Leu
290 295 300

Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His Leu

305 310 315 320

Phe Ser Thr Met Pro His Tyr Asn Ala Met Glu Ala Thr Lys Ala Ile
325 330 335

Lys Pro Ile Leu Gly Asp Tyr Tyr Gln Phe Asp Gly Thr Pro Trp Tyr
340 345 350

Val Ala Met Tyr Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro Asp
355 360 365

Arg Glu Gly Asp Lys Lys Gly Val Tyr Trp Tyr Asn Asn Lys Leu
370 375 380

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<213> Glycine max FAD2-1

<400> 33

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20 25 30

Lys Pro Pro Phe Thr Val Gly Gln Leu Lys Lys Ala Ile Pro Pro His
35 40 45

Cys Phe Gln Arg Ser Leu Leu Thr Ser Phe Ser Tyr Val Val Tyr Asp
50 55 60

Leu Ser Phe Ala Phe Ile Phe Tyr Ile Ala Thr Thr Tyr Phe His Leu
65 70 75 80

Leu Pro Gln Pro Phe Ser Leu Ile Ala Trp Pro Ile Tyr Trp Val Leu
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00436

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/29, 15/53, 9/02; A01H 1/00, 5/00, 5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (WPIDS) AND CHEMICAL ABSTRACTS - KEYWORDS BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GENBANK, EMBL, SWISSPROT, GENPEPT, PIR, TREMBL, WPIDS, CA, MEDLINE. Keywords: desaturase, de-saturase, cotton, gossypium, transgen?, transform?, genetically, modif?, engineer?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	The Plant Journal, vol. 13(2), 1998, Broun et al., "A bifunctional oleate 12-hydroxylase: desaturase from *Lesquerella fendleri*", 201-210	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	The Plant Cell, vol. 10, 1998, "Ribozymes Targeted to Stearoyl-ACP 9 Desaturase mRNA Produce Heritable Increases of Stearic Acid in Transgenic Maize Leaves", Merlo et al., 1603-1621	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	EP 561569 A (THE LUBRIZOL CORPORATION) 22 September 1993	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 June 2001	14 June 2001
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **CHRISTOPHER LUTON** Telephone No : (02) 6283 2256

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00436

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 945514 A (E.I. DU PONT DE NEMOURS AND COMPANY) 29 September 1999	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	WO 99/50430 A (DOW AGROSCIENCES LLC) 7 October 1999	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	WO 97/10328 A (RIBOZYME PHARMACEUTICALS, INC.) 20 March 1997	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	WO 91/18985 A (E.I. DU PONT DE NEMOURS AND COMPANY) 12 December 1991	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	US 5443974 A (HITZ et al.) 22 August 1995	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	US 5760206 A (HITZ et al.) 2 June 1998	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	US 5856157 A (CRAIG et al.) 5 January 1999	1, 5, 10-15, 18-24, 27-37, 46-52 1-38, 46-53
X Y	GenBank abstract accession number X95988, 6 March 1996, Liu et al.	53 1-38, 46-53
X Y	GenBank abstract accession number AJ132636, 15 February 2000, Liu et al.	53 1-38, 46-53
X Y	GenBank abstract accession number X97016, 29 October 1997, Liu et al.	53 1-38, 46-53
X Y	GenBank abstract accession number AJ244923, 26 May 1999, Brubaker et al.	53 1-38, 46-53
A	Current Opinion in Plant Biology, vol. 2, 1999, "Plant desaturases: harvesting the fat of the land", Napier et al., 123-127	1-60

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU01/00436

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	561569	AU	35167/93	CA	2092661	JP	6014667
		US	5777201				
EP	945514	NONE					
WO	9950430	AU	33678/99	EP	1068342		
WO	9710328	AU	67617/96	BR	9610402	CA	2226728
		CN	1196091	EP	842286		
WO	9118985	AU	79009/91	BR	9106506	EP	537178
		US	5443974	US	5760206		
US	5856157	NONE					

END OF ANNEX

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